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Recombinant Chromosome Substitution Lines as a source of genetic variation for drought stress tolerance in barley

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Recombinant Chromosome Substitution Lines as a source of genetic variation for drought stress tolerance in barley

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Declaration

I declare that I am the author of this PhD thesis and that the results presented here are of investigations conducted by myself. All references cited have been consulted by me and referenced accordingly to relevant researchers and their publications. The work presented here is my own and has not been submitted in candidature for any degree at any other university.

This is to certify that Carla de la Fuente Cantó has fulfilled the ordinances and regulations of The University of Dundee, so that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

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List of Abbreviations

13	2013
14	2014
2D	Two-dimensional
AB	Advanced Backcross
AMMI	Additive Main effects and Multiplicative Interaction
BLAST	Basic Local Alignment Search Tool
BLUEs	Best Linear Unbiased Estimates
BOPA	Barley Oligo Pool Assay
bp	base pair
BY	Biomass Yield
COL	Collar Height
Chr	Chromosome
cM	centimorgan
DAS	Days After Sowing
DII	Drought Intensity Index
DR	Drought (non-irrigated treatment)
DTI	Drought Tolerance Index
DY	Dry Yield
EAR	Ear length
FI	Full Irrigation treatment
GCP	Generation Challenge Programme
GE	Genotype by environment
GLS	Glossy spike
GO	Gene Ontology

GS	Growth Stage
HEA	Heading date
HEI	Total Height
HI	Harvest Index
IBSC	International Barley Sequencing Consortium
IPCA	Interaction Principal Component Axes
LSD	Least Significance Difference
M	Marker effect
MAS	Marker Assisted Selection
Mbp	Mega base pairs
M x **T**	Marker-treatment interaction effect
NIL	Near Isogenic Line
OSU	Oregon State University
PCA	Principal Component Analysis
PdE	Peduncle Extrusion
PdL	Peduncle Length
PI	Partial Irrigation treatment
QTL	Quantitative Trait Loci
RCSL	Recombinant Chromosome Substitution Line
REML	Residual (or restricted) maximum likelihood method
ROI	Region of Interest
RP	Relative Performance
SdA	Seed Area
SdL	Seed Length
SdW	Seed Width

SE	Standard Error
SHT	Seed shattering
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
TGW	Thousand Grain Weight
THR	Grain threshability
TILL	Number of tillers
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WS	Water stress
WSC	Water Soluble Carbohydrates
WUE	Water Use Efficiency
WW	Well-watered

Abstract

Exploring the naturally occurring genetic variation of the wild barley genepool has become a major target of barley crop breeding programmes aiming to increase crop productivity and sustainability in global climate change scenarios. However this diversity remains unexploited and effective approaches are required to investigate the benefits that unadapted genomes could bring to crop improved resilience. In the present study, a set of Recombinant Chromosome Substitution Lines (RCSLs) derived from a cross between an elite barley cultivar 'Harrington' used as the recurrent parent, and *Hordeum vulgare* subsp. *spontaneum* accession (Caesarea 26-24) from the Fertile Crescent, as the donor parent, have been utilised in field and controlled conditions to examine the contribution of wild barley genome as a source of novel allelic variation for the cultivated barley genepool.

Field evaluations in rain-out shelter over two growing seasons and contrasting water regimes revealed wide genetic variability among the RCSLs for relevant morphological, developmental and agronomic traits. Despite the generalised diminished performance of the RCSLs as compared to the elite parent, these were found to significantly improve grain weight and favour broader stability of this trait. The high-throughput genotypic characterisation of the lines with over 1,800 SNP polymorphic SNPs (Infinium iSelect 9K SNP chip), allowed QTLs associated with phenotypic variation to be identified using a mixed model approach. The study revealed novel QTLs for which candidate genes with putative effects on the mobilisation and accumulation of photo-assimilates during grain filling, could potentially lead to genetic gains in yield by optimising the crop sink strength. Also, QTLs associated with traits conferring adaptation to drought-prone environments such as plant cuticular waxes were identified. In addition, a two-dimensional paper growth pouch experiment revealed genotypic variation for root traits such as seminal root elongation rate, root diameter and gravitropism that could potentially determine differences in soil water and nutrient acquisition from early stages of development in restrictive environments.

This study has highlighted the role of exotic germplasm to contribute novel allelic variation by using an optimised experimental approach focused on an exotic genetic library. The results obtained constitute a step forward to the development of more tolerant and resilient varieties. Further investigation in groups of near isogenic lines need to be conducted to confirm the results of this experiments and narrow down the genetic basis of traits conferring adaptability to the crop.

1 General introduction

1.1 *Hordeum vulgare* L. brief overview

1.1.1 Global barley production and uses

Barley (*Hordeum vulgare* subsp. *vulgare* L.) was one of the earliest crops domesticated by the first Neolithic agrarian societies in the Near East and today it ranks fourth among the cereals in terms of total world production. In 2014, around 49.6 million hectares of barley were harvested worldwide, producing 144 million tonne of grain at an average yield of 2.9 tonne/ha. This was 5.1% of the world's total cereal produced after maize (36.5%), rice (26.5%) and wheat (26.0%), and more than half of the production (64.9%) and area harvested (51.0%) was in Europe (FAOSTAT, 2014).

Barley grain is mainly used for feed (55–60%) and processed into malt (30–40%) as a prime ingredient for beer and whisky production. Nowadays, its use as a food grain is minor (2–3%) compared to its origins as a crop, however it constitutes an important staple foodstuff in areas of Asia (Nepal, Tibet, Yemen), Africa (Morocco, Ethiopia, Eritrea) and the Andean region in South America (Ullrich, 2010). The stability of its yield compared to other cereals and its wider adaptability makes barley an important crop to contributing to food security in less favourable areas (Newton *et al.*, 2011). Additionally, the potential nutritional benefits of barley-based foods, as a source of β-glucans, have recently increased the interest for incorporating barley grain in people's diet (Brennan and Cleary, 2005). Finally, the utilisation of barley straw residues, as a sustainable biofuel feedstock, has become a new focus for the energy crops industry (Glithero *et al.,* 2013).

1.1.2 Barley taxonomic position

Hordeum vulgare L. first botanical description was provided by Linnaeus in the *Species Plantarum* of 1753. It is a grass belonging to the tribe Triticeae within the family Poaceae, the second largest family within the monocotyledon group. The tribe includes several economically important plants, such as wheat (*Triticum* spp.), rye (*Secale cereale*) and other forage grasses. The genus *Hordeum* harbours about 33 species dispersed across Eurasia, Africa, Central and South America (von Bothmer *et al.*, 1991) and its main characteristic is the typical "triplet"

which are three single-flowered (one seeded) spikelets at each rachis node (von Bothmer and Komatsuda, 2010).

Today, two barley subspecies are considered: *Hordeum vulgare* subsp. *vulgare*, which comprises cultivated barleys and *Hordeum vulgare* subsp. *spontaneum* or wild barleys of which brittle rachis, shrunken small weight seeds and allelopathic effect on the germination are the main differences with the cultivated barley (von Bothmer and Komatsuda, 2010). Depending on the number of fertile and developed florets at each node of the rachis, two botanical types can be distinguished: two and six row barleys (Fig. 1.1). The central florets are fertile in two row barleys, showing two rows of florets, one at each node of the rachis, while in the six row barleys, not only the central florets, but also the lateral ones are fertile, showing three florets at each node of the rachis.

Figure 1.1 Wild (*H. vulgare* subsp. *spontaneum)* and domesticated (*H. vulgare* subsp. *vulgare*) barleys spikes and spikelets in one rachis node. A) Wild barley spike showing brittleness of the rachis. B) Six-row (left) and two-row (right) cultivated barley spike. C) Wild barley spikelet triplet with thin-pointed lateral spikelets. D) Six-row barley spikelet triplet with three fertile, awned and filled with grain, spikelets. E) Two-row barley spikelet triplet with central spikelet, fertile, awned and filled with grain and two lateral infertile spikelets.

(A and B adapted from: *C Feuillet, P Langridge and R Waugh, Trends Genet (2008) 24:24-32*; C, D, and E adapted from: *M Pourkheirandish and T Komatsuda, Ann Bot (2007) 100:999-1008*)

In addition, barley can be distinguished by differences in growth habit into winter and spring types. These two forms are characterised by the differential responsiveness to two environmental cues: temperature and photoperiod (Karsai *et al.*, 2013). In winter barleys the transition to reproductive growth is promoted under long photoperiods (photoperiod responsive or sensitive) and its responsiveness will be favoured if the vernalisation requirements, or period of cold stimulus, are fulfilled (vernalisation responsive). This growth habit is generally present in wild barleys and it is considered as the ancestral adaptive mechanism to adjust flowering period to the most favourable season as is commonly seen in Israeli wild barleys (Nevo and Chen, 2010). On the other hand, the spring phenotype does not require a period of cold, although generally lacks frost tolerance. Spring barleys are therefore sown in spring and flower later being able to benefit from long mild summers.

It should be noted that, since wild barley and cultivated barley are interfertile, *Hordeum vulgare* subsp. *spontaneum* can be used to increase the genetic diversity of cultivated barley by crossing (Nevo and Chen, 2010). Furthermore, in terms of genetics, genomics and breeding, barley is considered as a model plant for the study of other crop plant species in the Triticeae tribe, since the gene loci in barley are largely collinear with the loci in other members of this group (Spannagl *et al.*, 2013). In addition to this, barley is widely used as an experimental organism due to its diploid nature (2n= 2x= 14) and self-fertility.

1.1.3 Origin and distribution of the crop

Barley (*Hordeum vulgare* subsp. *vulgare* L.) was one of the founder crops originating in the Fertile Crescent from its wild ancestor, *Hordeum vulgare* subsp. *spontaneum*. The Israel–Jordan region is considered the primary centre of domestication of the crop about ten thousand years ago (Fig. 1.2) (Badr *et al.*, 2000; Salamini *et al.*, 2002). However, phylogenetic studies of domestication traits leading the transition from wild to cultivated barley have also pointed to southern Central Asia as a secondary centre of origin, where domestication took place about 7000–8000 years ago (Morrell and Clegg, 2007; Saisho and Purugganan, 2007). The appearance of non-brittle rachis in cultivated barley, for example, seems to have arisen from natural mutations from wild barley in independent domestication events in eastern and western barleys (Komatsuda *et al.*, 2004; Azhaguvel and Komatsuda, 2007). Similarly, other domestication traits such as the hulled-naked caryopsis (Taketa *et al.*, 2004) and the spikelet row type (Komatsuda *et al.*, 2007) seem to have independent origins supporting a multicentre origin for the crop. Nevertheless, barley domestication and dispersion has been the focus of intense debate and different hypotheses supporting the polyphyletic origin of the crop have been proposed.

Regarding row type, initially two and six rowed domesticated barleys were classified as two different species, *Hordeum distichum* L. and *Hordeum hexastichum* L., but archaeobotanical studies have shown that both forms derived from common wild two-rowed ancestor (Pourkheirandish and Komatsuda, 2007). The two-rowed phenotype with brittle rachis constitutes an adaptive mechanism that facilitates seed dispersal in wild barley natural habitats and seed projection into the ground due to the arrowed shape of the reduced lateral spikelets. The loss of function of the *SIX-ROWED SPIKE 1* (*HvVRS1*) gene was due to single and independent mutations that occurred during or soon after domestication, turning the rudimentary lateral spikelets of two-row barley into fertile ones in the six-rowed barleys (Komatsuda *et al.*, 2007). Yet, the finding of a six-rowed barley with brittle rachis that grows wild in the Tibetan plateau, *Hordeum agriocrithon* (Åberg, 1938), questioned the common origin of both forms of barley and pointed to Tibet as an independent centre of origin of the crop (Ren *et al.*, 2013). However, *H. agriocrithon* may not represent a truly wild barley but is the result of natural hybridisation between a wild and a six-rowed cultivated form (Tanno and Takeda, 2004; Igartua *et al.*, 2013). Therefore, even though the six-rowed phenotype might have arisen in different domestication events in two-rowed ancestral barleys, it is unclear the role of Tibet as an independent centre of origin of the crop. Nonetheless, the distinctive genotypic variation in wild and domesticated germplasm from this region provides evidence for its significant value for crop diversification. Similarly, the genetically distinct wild and landrace barleys found in other centres of diversification of the crop such as Ethiopia and Morocco have also suggested that these areas are centres of origin of the crop (Molina-Cano *et al.*, 2005; Orabi *et al.*, 2007; Igartua *et al.*, 2013).

Figure 1.2 Map of the Near East. The green shaded area on the map highlights the area known as the Fertile Crescent, the primary centre of domestication of the *Triticeae* cereals ~ 10 000 years ago.

(Adapted from: *C Feuillet, P Langridge and R Waugh, Trends Genet (2008) 24:24-32*)

From the main two centres of origin of the crop, barley spread south into the Horn of Africa, north to Scandinavia, west towards the Mediterranean area and east into Asia (Kilian, Martin and Salamini, 2010; Dawson *et al.*, 2015). Established wild barley populations seem to have contributed locally adaptive variation to landraces during the gradual spread of the crop (Russell *et al.*, 2011). This way, western wild barleys appear to have contributed in the establishment of European and African landraces, whereas eastern wild barleys contributed most of the diversity in barleys from Central Asia and Far East (Fig. 1.3) (Morrell and Clegg, 2007; Saisho and Purugganan, 2007; Poets *et al.*, 2015).

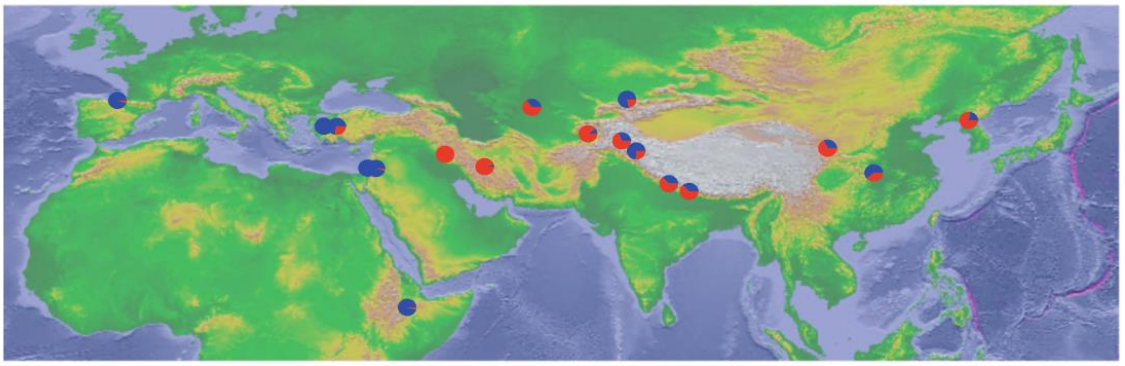

Figure 1.3. Estimated contribution of western (blue) and eastern (red) wild barleys to the diversity of cultivated genepool. Domestication in Fertile Crescent contributed the majority of the diversity of European and American barleys while the secondary centre of origin in Central Asia contributed most of the diversity of Asian and Far East cultivated forms.

(Adapted from: *P Morrell and M Clegg, PNAS (2007) 104: 3289-3294)*

Independent of the number of domestication events, today domesticated barley grows in a wide range of environments under a varied spectrum of climatic and input agricultural systems. It is cultivated both in highly productive agricultural systems and also in marginal and subsistence environments, where locally adapted cultivars or landraces play a significant role in a food security context. The broad geographic distribution of this ancient crop reflects not only the wide adaptive response of cultivated barley but also its importance for local and worldwide economies (Newton *et al.*, 2011).

1.1.4 Barley genetic diversity and adaptation

Barley grows today in a varied range of environments exposed to diverse climatic conditions from the semi-arid areas in the Middle East to the cold habitats in Tibet and the Andes (Nevo and Chen, 2010; Paulitz and Steffenson, 2011). The rather versatile condition of the species is quite remarkable compared to other small grain cereals and their wild ancestors and appears to be much more salt and drought tolerant (Nevo and Chen, 2010). In fact, it has been described as the "last crop before the desert" since wild barley populations grow well in harsh

environmental conditions on the edge of the deserts in the Fertile Crescent and West Asia. This broad demographic distribution reflects the adaptiveness of the crop to a variety of growing conditions.

During the last glacial maximum (21,000 years ago), wild populations found refugial areas in the eastern Mediterranean region and central Asia, in locations considered today as hot-spots of genetic diversity (Russell *et al.*, 2014). Predictive models have aided the identification of these refugia regions as well as current natural habitats for the species that might be at risk in a mid-term future due to changes in climatic variables. Northern Iran, southern Syria and Turkmenistan constitute interesting targets not only for germplasm conservation but also to monitor adaptation in response to anthropogenic climate changes (Russell *et al.*, 2014; Dawson *et al.*, 2015). Either differential ecotypes with diverged phenotypes might arise as a consequence of adaptive processes such as genetic drift, similar to the adaptation observed in Israeli desert and Mediterranean wild barleys (Hübner *et al.*, 2013), or migration of the population to more suitable habitats, which would define new areas of the species.

The gradual process of selection in the transition from ancestral wild barleys to locally adapted varieties and elite cultivars has reduced significantly the genetic diversity of the crop compared to its wild progenitor (Russell *et al.*, 2011). Indeed, it has been estimated that only about 40% of wild barley alleles are represented in the cultivated barley gene pool (Ellis *et al.*, 2000; Russell *et al.*, 2000). Genetic bottle-necks occurred immediately post-domestication and through modern breeding practices based on inbreeding and "breeding the best with the best" have dramatically narrowed the crop genetic basis for future crop improvement (Fig. 1.4) (Tanksley and McCouch, 1997; Meyer and Purugganan, 2013). However, this process has aided the fixation of allelic combinations for desirable domestication-related traits in the crop. For example, diversity studies carried out in different groups of genotypes within the barley primary gene pool (wild, landraces and cultivars) have shown that reduced genetic diversity occurs in the cultivated forms for key domestication loci such as non-brittle rachis *btr1/btr2* (Comadran *et al.*, 2011), the *vrn1* locus responsible of the six-rowed phenotype (Igartua *et al.*, 2013), and the *nud* locus related to the naked caryopsis (Taketa *et al.*, 2004). Interestingly, the genomic analysis of 6,000-year-old barley grains of landraces from the Judean desert revealed the early fixation of some of these loci and its relevance in the domestication process of the species (Mascher *et al.*, 2016). However, selection favouring advantageous allelic combinations for crop production has significantly reduced the genetic diversity at flanking and neutral loci linked to the target domesticated ones as a consequence of linkage drag or the tendency of close alleles to be inherited together (Kilian *et al.*, 2006; Jakob *et al.*, 2014). Less significant but also noticeable is the loss of genetic diversity around non-domesticated genes (Yan *et al.,* 2015).

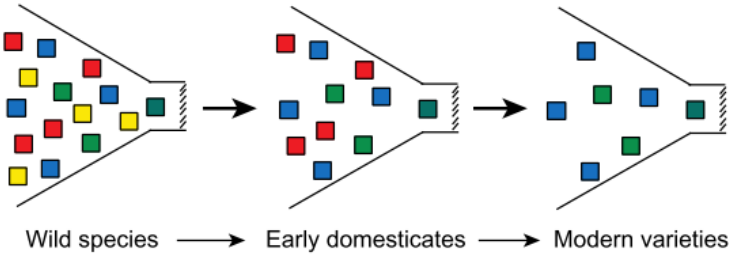

Figure 1.4 Genetic bottle-necks occurred on crop plants as a consequence of the domestication process and the selection through modern breeding practices. Allelic variations of genes are represented with different coloured boxes showing the gradual loss of genetic diversity in modern crop varieties

(Adapted from: *S Tanksley and SR McCouch, Science (1997) 277:1063-1066)*

The reduction in genetic diversity in the transition from wild barley to cultivated forms has recently been revealed in a genome-wide analysis of protein-coding genomic regions, or exome capture analysis (Mascher *et al.*, 2013b), using a large representation of adapted and unadapted barleys (Russell *et al.*, 2016). Not only were signatures of selection identified for the key domestication-genes, but also twelve-fold fewer rare allelic variants (with a minor allele frequency below 5%) were detected in the landrace genotypes compared to the wild barleys illustrating the effect of domestication bottle-necks. Additionally, differential allelic variation patterns were found associated with environmental variables suggesting differences in the adaptive responses in the two groups of germplasm and the genes involved in the expansion of the species and establishment of the crop. A good example of this would be the natural variability found for *HvCEN* and its relationship with variation in flowering time conferring adaptation in the latitudinal expansion of the species (Comadran *et al.*, 2012). Different allelic variants of the gene seem to have contributed to the gradual transition from the winter to the spring growth habit. Selection pressures for late flowering patterns in the north European spring barleys seem to have fixed the gene haplotype associated with the later flowering phenotype which is advantageous for yield production in higher latitudes. However the genetic diversity of the surrounding genomic region in this gene pool is presumably reduced as a consequence of selection.

Rare allelic variants prevailing in wild germplasm constitute a highly valuable genetic resource for broadening the genetic basis of the crop and increase its adaptability to less favourable or changing environmental conditions (Feuillet *et al.,* 2008; Warschefsky *et al.*, 2014). New breeding strategies are being exploited in order to explore novel allelic variation lost through the breeding process of the crop, which has been predominantly conducted for selecting high yielding lines in productive agricultural systems, and therefore biased against the selection of lines with superior stress tolerance (Forster *et al.*, 2000).

1.1.5 Wild and landrace barley germplasm for crop improvement

Traits favouring yield stability under harsh environmental conditions have become new desirable breeding targets to face future climate challenges and pressures of a globally growing population (Powell *et al.,* 2012). Even though improving barley productivity is still achievable through breeding programmes focused on elite germplasm (Thomas, 2003), the rich genetic diversity harboured in the undomesticated germplasm and locally adapted varieties constitutes a reservoir of new allelic variation for broadening the genetic base of the crop, especially towards the development of climate-resilient crops (Ellis *et al.*, 2000; Dawson *et al.*, 2015).

The natural genetic diversity of the species is being preserved in large *ex situ* collections of barley germplasm. The FAO *Second Report on the State of the World's Plant Genetic Resources* (FAO, 2010) estimates that 466,531 barley accessions are present in genebank collections throughout the world, being the third crop worldwide with the largest *ex situ* collection after wheat (856,168) and rice (773,948). In a previous report, the Global Crop Diversity Trust (www.croptrust.org) estimated that 15% of the genetic resources preserved in barley collections corresponded to wild accessions and 44% to landraces. The rest, 42%, corresponded to breeding lines (17%), cultivars (15%) and genetic stocks (9%) (Global Crop Diversity Trust, 2008).

Some of these resources have been used in diversity studies showing the considerable natural variation existing in wild and landrace barleys for genes involved in abiotic and biotic stresses that constrain crop production. For example, novel genetic diversity for cold (Fricano *et al.*, 2009) and heat (Xia *et al.*, 2013) tolerance as well as for genes favouring adaptation to droughted (Kilian *et al.*, 2006; Suprunova *et al.*, 2007) and high salt (Wu *et al.,* 2011) environments have been attributed to this gene pool. Also rich genetic variation for disease resistance genes has been associated with exotic genomes (Fetch *et al.,* 2003).

Quantitative genetic studies have reported the potential benefits of this natural genetic variation in different adapted genetic material. Elite lines have been found to benefit from exotic alleles for increased resistance to common foliar diseases, such as leaf rust, spot blotch and scald (von Korff *et al.*, 2005; Li *et al.*, 2006; Yun *et al.*, 2006; Hofmann *et al.*, 2013) and increased tolerance to abiotic stresses such as drought and salt (Kalladan *et al.,* 2013; Honsdorf *et al.,* 2014a, 2014b).

However, the main challenge of the "rewilding" process entails the introduction of exotic favourable alleles associated with natural plant adaptability into elite modern cultivars while crop yield potential and quality is preserved (Palmgren *et al.*, 2014). In the last twenty years,

hybridisation between crops and wild relatives has been achieved through introgression breeding or crosses performed between an elite genotype and genetically distant wild accessions or landraces. During this time, wild relatives' genetic diversity has been proved to enhance productivity and performance of some crop species, in particular wheat and rice within the group of cereals crops (Hajjar and Hodgkin, 2007). In barley, one of the first examples is increased resistance to powdery mildew achieved in the European spring cultivars in the 1980s due to the introgression of the *mlo-11* gene associated with natural resistance in Ethiopian barley landraces. This gene successfully provided increased resistance in commercial cultivars for more than thirty years (Jorgensen, 1992; Feuillet *et al.,* 2008). More recently, as part of the research programmes carried out in the International Centre for Agricultural Research in Dry Areas (www.icarda.org), wild barley accessions have been successfully used in the development of breeding lines with increased adaptation to drought stress in Mediterranean environments (Hajjar and Hodgkin, 2007; Lakew *et al.*, 2011). An undomesticated barley accession from the Fertile Crescent used in crosses with a landrace was found to increase grain yield by 50% compared to other barley cultivars in dryland conditions (Ashraf, 2010). However there are only a few examples, since the exotic genetic diversity remains unexploited in breeding programmes (Zamir, 2001).

Although hybridisation with wild relatives is useful for improving adaptability of the crop, introgression breeding still presents some limitations to be used routinely in breeding programmes. Lines derived from wide crosses tend to perform poorly in relation to the elite parent since alien chromosome regions from undomesticated germplasm generally introduce deleterious genes linked to the desirable ones (Feuillet *et al.,* 2008; Warschefsky *et al.*, 2014). However, effective marker assisted selection and several backcrosses with the elite variety help to maintain some of the agronomic potential of the crop and also select against the deleterious wild characters in the population (von Korff *et al.*, 2007; Schmalenbach and Pillen, 2009). In addition, the establishment of exotic genetic libraries through introgression breeding has been proposed as an effective first step towards the identification of exotic target genes to include in breeding programmes (Zamir, 2001). Groups of lines with marker-defined genomic regions of a wild accession in the same cultivated elite genetic background allow screening of traits harboured in wild genomes and evaluate its putative agricultural value under different experimental conditions. These permanent genetic resources have been found to be useful for exploring the potential of natural genetic diversity in the improvement of crops such as wheat (Huang *et al.*, 2003), maize (Mano and Omori, 2013) and rice (Fukuoka, Nonoue and Yano, 2010). In barley, groups of introgression lines derived from an initial cross between an elite cultivar and wild accession (Matus *et al.*, 2003; Pillen, Zacharias and Léon, 2003; von Korff *et al.*, 2004; Hori *et al.*, 2005; Schmalenbach *et al.,* 2008) have aided the identification of

chromosome regions and genes for which the exotic variants could increase crop performance (see section 1.3.4.1).

1.2 Drought stress and crop production

1.2.1 Drought impact on agriculture

Drought in agriculture is understood as a condition in which the water available is insufficient to meet the transpiration needs of the crop (Tuberosa, 2012). This limitation causes a suboptimal performance of the crop which impacts on yield production which are dependent on the timing, duration and intensity of the water scarcity (Chaves and Oliveira, 2004; Cattivelli *et al.*, 2008). Drought constitutes the major constraint for crop and food production and its effects are likely to increase in global climate change scenarios (Dai, 2011; Olesen *et al.*, 2011). Frequency of long-term drought periods associated with El Niño episodes are expected to increase in regions already vulnerable such as the Horn of Africa, west part of the United States, India, northeast China, and New South Wales in Australia (FAO, 2014). Also, variation in patterns of precipitation, evaporation and soil moisture are projected to change considerably, with increased aridity over global land (Dai, 2011).

Today, agriculture uses approximately 70% of the water consumed worldwide and 40% of world food supply depends on irrigated soils (Chaves and Oliveira, 2004). The pressure of a growing population on the water and land resources is already challenging the current situation, "*more crop per drop*" production has become the main slogan for different research and breeding programmes. Both, breeding strategies to develop more tolerant crops and improvement of agronomy practices are required to optimise the available resources and maintain productivity within changing environments (Passioura, 2006a; Powell *et al.*, 2012).

Cereal crops are the main staple for human food and animal feed and their grain production and yield stability are highly affected by environmental constraints, particularly water scarcity (Araus *et al.*, 2002). Limited water supply during flowering time, for instance, can cause major impacts on yield production since this is the most drought stress sensitive growth stage, the regulation of which is critical for conferring adaptation to the environment (Passioura, 2007; Tuberosa, 2012; Campoli and Von Korff, 2014). Consequently, rain-fed agricultural systems are particularly susceptible to seasonal drought periods. Even in climate favourable regions of central Europe, spring cultivars have been significantly affected by variation in rainfall patterns during the crop growing season compared to winter cultivars, resulting in financial losses (Hlavinka *et al.*, 2009; Olesen *et al.*, 2011). More worrying is the alteration in rainfall patterns occurring in countries like Ethiopia, where cereals such as maize, teff, sorghum and barley are particularly important for food security and constitute the major food crops. Erratic distribution

of seasonal precipitation and seasonal shifts in rainfall patterns are causing short and long-term droughts difficult to predict, which generally results in crop failure (Evangelista *et al.,* 2013). In this context, investigating plant adaptation mechanisms for coping with current and future environmental constraints is critical for developing more tolerant crops. In conjunction, accurate climatic predictive models are also needed to aid the selection of suitable crops or cultivars for each growing season as well as to define the optimum timing for cultivation.

1.2.2 Plant adaptation and responses to water deficit

1.2.2.1 Plant tolerance mechanisms

Morphological changes increasing water uptake (e.g. by the development of improved root systems) or reducing water loss through the aerial plant organs (e.g. by and improved regulation of stomatal closure and transpiration or the accumulation of protective cuticular waxes) facilitate drought avoidance or reduction of tissue water deficits under stress (Shepherd and Griffiths, 2006; Barbour *et al.*, 2010; Aroca *et al.,* 2012). Also, modifications in the growing cycle can allow the plant to escape from droughted periods by adjusting flowering time to the most favourable conditions (Passioura, 2007). In addition, physiological osmoprotective functions occurring at the cellular level, such as the accumulation of compatible solutes in the cytoplasm as well as an improved antioxidant defence, would maintain cell turgor and photosynthesis under low water potentials (Chaves and Oliveira, 2004). Yet, the caveat to most of these adaptations is that even if they favour survival in drought stress periods, they also tend to reduce productivity, an undesirable trait for crop plants (Blum, 2005; Tuberosa, 2012).

Traits such as the adjusted plant phenology, the cuticular waxes and the extended root system are mostly constitutively expressed rather than stress induced. In contrast, effective regulation of stomatal conductance or active osmoprotective functions occurring at cellular level are promoted by complex signalling pathways regulated by plant hormones like abscisic acid (ABA) which are triggered in response to changes in plant water status (Chaves, Maroco and Pereira, 2003; Cattivelli *et al.*, 2008).

1.2.2.2 Yield under drought

The effects of drought on yield would depend on the adjustments in dry matter partitioning at the whole plant level. The translocation of carbon photo-assimilates produced in the source organs (mainly leaves) into the reproductive sink organs (grains) determines the reproductive development of the plant and therefore, seed yield (Farooq *et al.*, 2009).

Under environmental constraints such as drought, plants readjust the balance between source and sink organs to optimise the reproductive fitness under stress. In crops, these adjustments generally lead to significant yield reductions. However, compensatory effects in response to stress would determine plant plasticity and therefore its capacity to adapt yield under or upon water limited situations (Marcelis, 1996; Lemoine *et al.*, 2013). Yield components such as the number of grains produced, grain size and weight would be affected depending on the timing and duration of water shortage and the plant growth stage at which the stress occurs (González *et al.,* 2007). In this regard, different factors would influence source–sink relationships during the pre-anthesis and post-anthesis periods (Fig. 1.5). For example, reduction in growth and leaf expansion as a consequence of the stress would reduce leaf area and therefore gas exchange and sucrose accumulation for grain filling (pre-anthesis source reduction). Also, drought stress might stop new tillers forming or the death of the growing ones which would limit the seed set as a consequence of reduced spikelets and floral structure development in the pre-anthesis period (pre-anthesis sink reduction) (Coventry *et al.*, 2003). Therefore, water shortage at pre-anthesis contributes directly to yield reduction by limiting the number of ears and grain developed per unit land, having also an indirect effect on grain size and weight as a consequence of the diminished availability of assimilates for grain filling.

Drought at anthesis or during grain filling can cause early senescence of the leaves, limiting photosynthesis and formation of carbohydrates during the grain filling period (Coventry *et al.*, 2003; Barnabás *et al.,* 2008). In this case, the lack in current photo-assimilates formation would promote the contribution of remobilised carbohydrates stored in the pre-anthesis phase to grain filling which would mainly depend on this source of assimilates (post-anthesis source reduction). In addition, drought can induce sterility on male or female organs in the inflorescence at anthesis as well as abortion of embryos post-anthesis (sink reduction post-anthesis) (Tardieu, 2012). Also, differences in cell division and grain filling rate as well as the activity of starch synthesis enzymes in the endosperm would define the capacity of grain dry matter accumulation and therefore its size and weight (Zhang *et al.*, 2014). Hence, water deficit during post-anthesis phase contributes to yield reduction by limiting the number of viable seeds produced and their weight.

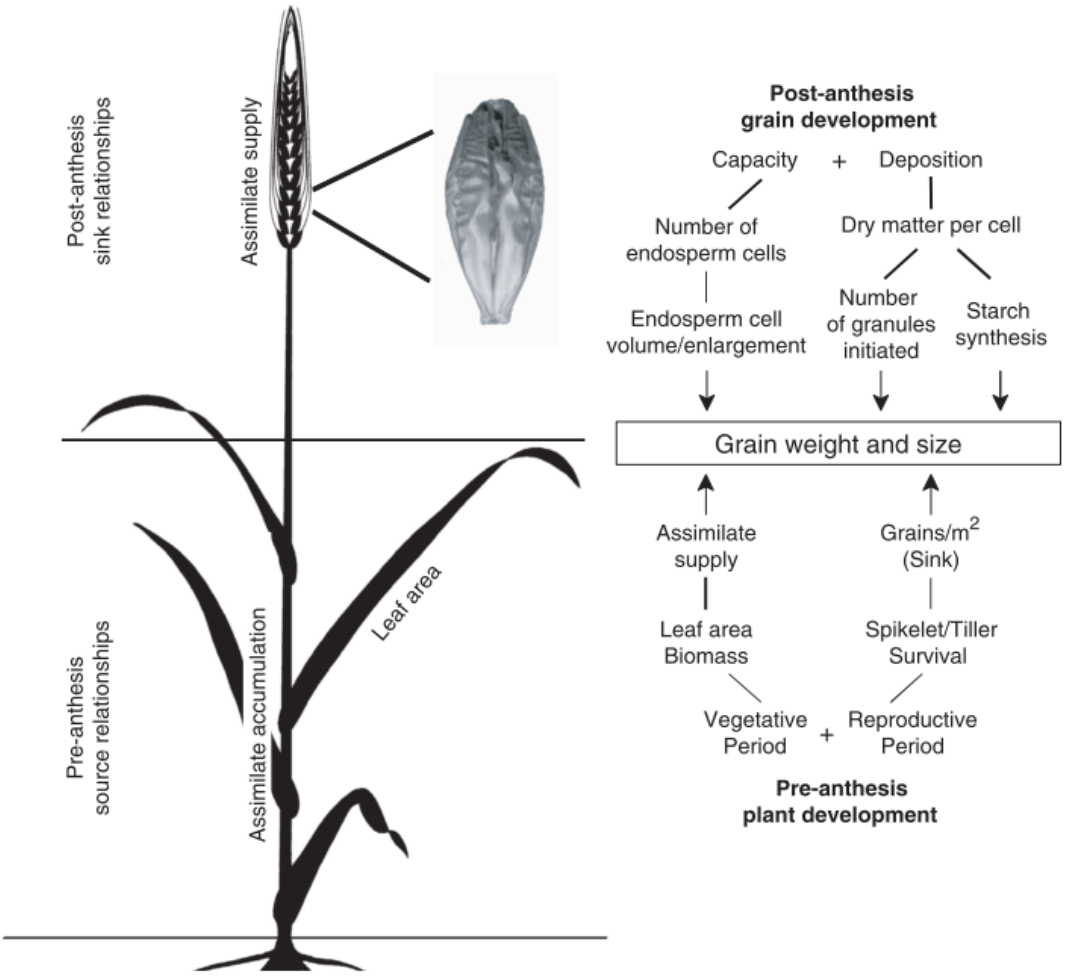

Figure 1.5 Main factors determining grain size and weight during pre- and post-anthesis periods of barley development. The pre-anthesis period influences grain size and weight indirectly by determining source availability and sink size. The post-anthesis period influences directly grain development and demand of photo-assimilates.

(Adapted from: *SJ Coventry, AR Barr, JK Eglinton and GK McDonald, Aust J Agric Res (2003) 54: 1103-1115*)

Genotypic variation in the partitioning and distribution of dry matter from source to sink organs has been shown to contribute genetic gains on yield production in target environments. For example, durum wheat genotypes with increased contribution of the carbon photo-assimilates stored in the stems during the pre-anthesis period showed an optimised dry matter translocation during grain development in Mediterranean environments (Álvaro *et al.*, 2008). In mild climatic conditions, genotypes with a longer pre-anthesis period and increased vigour would favour the development of more grains per unit land and build a larger source for grain filling in a non-stress post-anthesis period (Araus *et al.*, 2008).

1.2.3 Breeding for drought

Drought tolerance is a multi-genic trait. The response largely depends on the length, timing and intensity of the water deficit period (Farooq *et al.*, 2009). The mechanisms developed by drought tolerant plants to withstand the effects of water deficit would be the main interest for developing crops with enhanced drought resistance. However, drought is also the most complex abiotic stress to study since seasonal changes in water availability might occur with other environmental constraints for plant development such as increased temperatures, irradiance, salinity, or soil strength. The combination of stresses would modulate plant adaptive responses and also the impact on crop performance (Iijima and Kato, 2007; Barnabás *et al.,* 2008). In Mediterranean environments, for instance, cereal crops are at risk of suffering high temperatures and water deficit stresses at the end of the growing season which will impact on the grain filling phase (Voltas *et al.*, 1999; Francia *et al.*, 2013). Nevertheless, in some cases plant response mechanisms to abiotic stresses are closely related (Cattivelli *et al.*, 2002; Zhu, 2002).

From a breeding perspective, the main challenge is to develop cultivars that perform well under drought without losing the ability to yield well in favourable seasons (Passioura, 2012). In this regard, improving ratio of product yield to the amount of water 'used' would be the main objective and so, breeding for drought would focus on improving crop "water productivity" rather than on "drought tolerance" *per se* (Passioura, 2006a).

Increases in crops yield potential achieved through empirical breeding by selecting high yielding phenotypes in favourable conditions, has proved to be successful and contributed, to some extent, to improved yields under drought (Blum, 2005; Araus *et al.*, 2008). However, this approach appears to be quite restrictive for achieving further yield and also reduces the difference between yield under water-deficit conditions and actual yield potential (Blum, 2005; Araus *et al.*, 2008; Cattivelli *et al.*, 2008).

The quantitative nature of yield, its low heritability and large GE interaction require analytical breeding approaches based on the understanding of the physiology underlying the GE interaction and the screening of yield-determining secondary traits that could directly contribute genetic gains on yield (Araus *et al.*, 2008; Tuberosa, 2012; Malosetti *et al.,* 2013)

1.2.3.1 Measuring crop yield stability and adaptability

Generally, the stress level to which the plants have been exposed is associated with the impact that it has had on the yield production and therefore, it is measured by the decrease of grain

yield and quality, referred to as the yield potential, under favourable conditions (Blum, 2011). Some genotypes may be widely adapted, performing well across a wide range of conditions, whereas others may show specific adaptation to a delimited set of conditions. In this case the response is largely determined by the genotype by environment (GE) interaction, and crop performance will rely on variations in the environmental conditions (Cooper and Byth, 1996; Malosetti *et al.,* 2013). Broadly adapted cultivars have been the main target of breeding programmes since they show a superior and steady performance in a wider range of conditions, however breeding for specific adaptation is also required. Assessing the specific adaptation strategies associated with the GE interaction could complement traditional methodologies based on selection for broader adaptability (Fleury *et al.*, 2010). Different approaches are used in the identification of superior genotypes according to the stability in performance with respect to environmental changes in time and/or the adaptability across a range of different environments.

Numerous selection indices based on the mathematical relationship between yields under favourable and stress conditions have been suggested to rank groups of genotypes in regard to the relative impact of water deficit on their performance in relation to the mean effect (Sio-Se Mardeh *et al.*, 2006). For example, the drought susceptibility index proposed by Fischer & Maurer (1978) has been widely used for identifying drought tolerant and susceptible genotypes based on yield stability under drought (Shakhatreh *et al.*, 2001; Ober *et al.*, 2004; Inostroza *et al.*, 2007) as well as stability of grain quality traits (Kalladan *et al.*, 2013), biomass production (Abdel-Ghani *et al.*, 2015) and photochemical activity and gas exchange (Rapacz *et al.*, 2010). With this index, generally greater sensitivity corresponds to reduced stability (Szira *et al.*, 2008).

Several statistical analyses have been designed to effectively simplify the study of the GE interaction and evaluate yield adaptability in a range of different environments (Annicchiarico, 1997). The two most commonly used approaches are the joint regression analysis proposed by (Finlay and Wilkinson, 1963) and multivariate analytical methods such as the Additive Main effects and Multiplicative Interaction analysis (AMMI) (Gauch, 2006). The former is a single dimension analysis that defines yield stability as the slope of regression of yield for an individual line on the mean yield across environments and lines. This method has been widely used to assess GE interaction patterns in a group of genotypes and identify possible yield cross-over interactions or genotypes with a superior performance in response to the environment (Fig. 1.6) (Tambussi *et al.*, 2005; Francia *et al.*, 2013). The AMMI model examines the additive effect of GE interaction in a principal component approach, which may be a more flexible means for capturing a larger proportion of GE interaction (Malosetti *et al.,* 2013). The scores of the principal components for the different genotypes can be understood as stability parameters

(Lacaze *et al.,* 2009). This method has been found useful for identifying genotypes with broad adaptation in a range of environments tested as well as genotypes showing satisfactory performance consistently in a particular location (Rodriguez *et al.*, 2008; De Vita *et al.*, 2010).

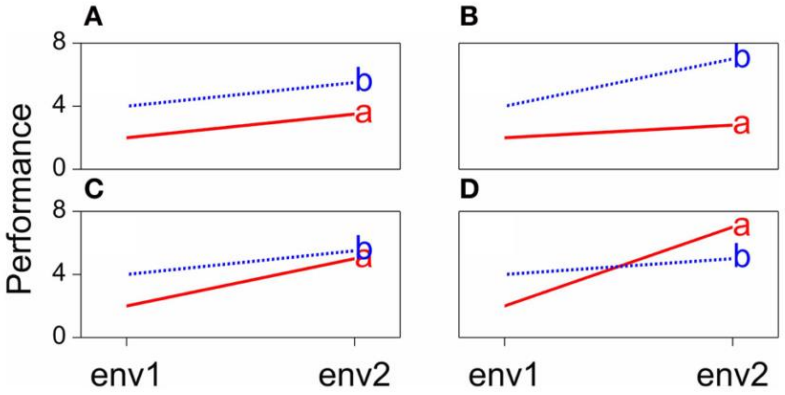

Figure 1.6 Theoretical framework of genotype-by-environment (GE) interaction illustrated as change in mean performance of two genotypes across environments. A) Additive model with no GE interaction. B) Divergence model. C) Convergence model and D) Cross-over interaction in which genotype performance is largely determined by the environment.

(Adapted from: *M Malosetti, JM Ribaut, Eeuwijk and F van Euewijk, Front Physiol (2013) 4: 1-17*)

1.2.3.2 Selection of secondary traits

Analytical breeding strategies for drought could focus on secondary traits which increase water use, water use efficiency (WUE) or harvest index (Araus *et al.*, 2002, 2003; Passioura, 2006a). The selection however will depend strongly on the target environment. For example, earliness may favour yields in environments exposed to terminal drought periods whereas it would be detrimental for maximising crop production in northern temperate regions (Acevedo *et al.*, 1991; van Oosterom and Acevedo, 1992; Shakhatreh *et al.*, 2001; Forster *et al.*, 2004; Cuesta-Marcos *et al.*, 2009). Also, improved water use efficiency may be beneficial in drought-prone regions that depend on stored soil water all through the crop growth cycle (Rebetzke *et al.*, 2002; Condon *et al.*, 2004). However it might be associated with slow growth and reduced crop performance in moderate or non-stressed environments (van Den Boogaard *et al.*, 1997; Condon *et al.*, 2004). According to Araus *et al.* (2008) a few aspects should be considered in the selection of secondary traits:

1. There must be a genetic correlation between the secondary trait and grain yield in the target environment.
2. The secondary trait should be less affected by the environment than yield.
3. There must be genotypic variation for the secondary trait within the species.

4. The selection of a secondary trait should be based on stress avoidance mechanisms rather than drought tolerance. If possible, it should not be associated with poor yields in favourable environments.
5. Measuring a secondary trait should be straightforward and provide a reliable score.
6. A secondary trait should be scorable in individual plants or small plots.

In cereals, secondary traits putatively related to crop performance such as plant height, flowering time, plant vigour, stomatal conductance etc. have been largely evaluated in breeding programmes for improving drought tolerance (Araus *et al.*, 2003; Cattivelli *et al.*, 2008). These are easy measurable characters with greater heritability than yield itself and with variation favouring plant adaptability and productivity in the target environments. Other traits such as carbon isotope discrimination, remobilisation of water soluble carbohydrates or root architectural traits have been less explored in breeding programmes due to the limitations derived from the cost and time required for their determination. However, these have been highlighted as promising traits for achieving additional genetic gains in yield, particularly in dry environments (Coventry *et al.*, 2003; Slafer *et al.*, 2005; Lynch, 2007). Different strategies can be addressed when breeding for larger yields and increased adaptation to certain level of water stress through the selection of secondary traits.

i. Adjusted plant phenology

The timing to reach flowering is a pivotal secondary trait contributing to plant adaptation to the environment and used in breeding programmes to adjust cultivars phenology to particular areas (Boyd *et al.*, 2003; Passioura, 2006a). Adjustments in crop phenology allow matching crop development to seasonal rainfall patterns, contributing to increase water use by the crop and so favouring water use efficiency at key growth stages (Richards *et al.*, 2002; Barnabás, Jäger and Fehér, 2008). It is an easy trait to score under field conditions and its genetic basis has been widely studied in several crop plants. In cereals, the transition from vegetative to reproductive phase is controlled by several known genes conferring earliness *per se* or responses to two main environmental cues: temperature and day length (Casao *et al.*, 2011; Comadran *et al.*, 2012; Rollins *et al.*, 2013; Tondelli *et al.*, 2013; Campoli and Von Korff, 2014).

ii. Balanced plant height and dry matter partitioning

The development of semi-dwarf elite cultivars during the “Green Revolution” readjusted the plant dry matter partitioning, favouring larger sinks aboveground and more grain produced per unit of biomass (Hedden, 2003). Improved harvest index is achieved at an optimised height (70–100 cm) in favourable environments, and no significant gains in yield are reached above or below this limit (Richards, 1992; Slafer *et al.*, 2005; Araus *et al.*, 2008). However, reduced plant height of modern varieties can be detrimental for crop establishment and production in

less favourable conditions, where increased plant height confers adaptability and favours harvest index and hence yield (Shakhatreh *et al.,* 2001; Rebetzke *et al.,* 2007; Rosyara *et al.,* 2009). Further increases in yield under drought through plant height adjustments are still possible, although this may be a complicated approach since modifications in plant height generally affect other important yield related traits associated with plant growth cycle and development such as flowering time and tiller development (Baum *et al.,* 2003; Talamè *et al.,* 2004; Gyenis *et al.,* 2007).

The contribution of stem reserves to grain development is considered as an important alternative and more effective secondary trait for achieving genetic gains in yield and harvest index, particularly under terminal drought conditions (Chaves and Oliveira, 2004; Slafer *et al.,* 2005; Araus *et al.,* 2008; Cattivelli *et al.,* 2008). An efficient remobilisation of water soluble carbohydrates (WSC) accumulated during pre-anthesis can mitigate the impact of water deficit during grain filling, especially when photosynthesis is impaired due to the stress (Bonnett and Incoll, 1993a, 1993b). Water soluble carbohydrates are actively mobilised in response to stress and large genotypic differences have been found (Blum, 1998, 2005; Ehdaie *et al.,* 2006). Stem reserve accumulation and mobilisation can be assessed easily in field grown plants by direct and indirect estimations of shading plant (Bonnett and Incoll, 1993a; Ehdaie *et al.,* 2006) or spraying chemical desiccants after anthesis (Blum, 1998; Salem *et al.,* 2007). It should be noted that the ability to accumulate large stem reserves to sustain grain filling under stress might exert losses in grain yield potential in modern cultivars since it is generally correlated with increased plant height and enhanced leaf senescence (Blum, 1998; Barnabás *et al.,* 2008).

iii. Improved Water Use Efficiency

Water use efficiency (WUE) refers to the aboveground dry mass produced per unit of water transpired which directly depends on the photosynthetic transpiration efficiency or amount of carbon fixed per unit of water transpired (Araus *et al.,* 2003). Both factors are known to be associated with greater yield under water deficit conditions (Barnabás *et al.,* 2008).

Secondary traits favouring increased water uptake or enhancing the transpiration efficiency through the leaf surface would contribute differences in WUE (Tuberosa, 2012). For example, a robust root system may enhance water uptake under stress favouring increased crop performance (Chloupek *et al.,* 2010). Also, increased early vigour enables greater water use and so seedling establishment by allocating soil evaporation into plant transpiration (Rosyara *et al.,* 2009). In contrast, a conservative control of stomata conductance would maintain plant water status and ameliorate WUE by reducing transpiration losses under drought (Samarah *et al.,* 2009). Likewise, a higher photosynthetic capacity contributes to improve WUE (Tambussi

et al., 2007). In addition, leaf anatomical traits such as the presence of epicuticular waxes contribute to enhance leaf reflectance, improving transpiration efficiency and WUE (Febrero *et al.*, 1998).

Under drought stress, the carbon isotope discrimination ($\Delta^{13}C$) has been found to be a reliable estimate for stomatal conductance, transpiration efficiency and WUE in C_3 species (Condon *et al.*, 2004). $\Delta^{13}C$ measures the ratio of stable carbon isotopes ($^{13}C/^{12}C$) in the plant dry matter compared to the ratio in the atmosphere, which results from the discrimination of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) against the heavier isotope, ^{13}C. As the leaf CO_2 concentration reduces with larger transpiration efficiency, the isotope discrimination against ^{13}C would decrease, thus the value of $\Delta^{13}C$ correlates negatively with transpiration efficiency (Farquhar and Richards, 1984; Condon *et al.*, 2004). The large genetic variation, large heritability and small GE interaction of $\Delta^{13}C$ (Rebetzke *et al.*, 2002; Barbour *et al.*, 2010), makes this trait a very interesting target for drought breeding programmes. However, its main drawback is that it is costly to measure (Araus *et al.*, 2003). An inexpensive alternative to estimate crop transpiration is measuring canopy temperature with an infrared thermometer which allows for larger throughput phenotyping and gives an indication of stomatal conductance. In this case, the reliability of the measurement can be confounded by the changes in the environmental conditions and it is more suitable for obtaining relative comparison in a set of genotypes instead of absolute measurements (Munns *et al.*, 2010).

1.2.3.3 Improved root system for increased water capture

Several studies have emphasised the importance of plant roots as target for new breeding programmes focused on getting more efficient and sustainable crops (de Dorlodot *et al.*, 2007; Lynch, 2007; Herder *et al.*, 2010). Their fundamental role in water and nutrient capture has direct effects on aboveground performance and therefore a large potential for crop improvement (Gregory *et al.*, 2009; Steele *et al.*, 2013). Genotypic differences in root system traits have been widely reported, and modifications in key root traits have been found to increase crop performance in water stressed environments (Steele *et al.*, 2013; Uga *et al.*, 2013). However, the complexity and inaccessibility of the root systems limit routine screenings for targeting favourable root traits to introduce in breeding programmes. Indirect measurements indicating a better water status aboveground (e.g. lower canopy temperature or an increased grain $\Delta^{13}C$) have been considered as estimates of an improved capacity to take up soil moisture (Slafer *et al.*, 2005; Araus *et al.*, 2008). Nevertheless, direct root phenotypic evaluations are required for identifying putative root-secondary traits contributing to crop improvement under drought.

Temperate cereal crops such as barley develop a fibrous root system composed of seminal and nodal (or adventitious) roots arising from the embryo and the base of the growing shoot respectively (Hackett, 1968). Seminal roots play an important role in extracting water from deep soil layers, especially under drought conditions, since they are the first to develop and they grow deeper in the soil profile than nodal roots (Grando and Ceccarelli, 1995; Knipfer and Fricke, 2011). These, in contrast, start developing from the three leaf growth stage and supply water to the crop mainly from the topsoil (Richards *et al.*, 2002). Primary roots (seminal and nodal) establish the overall plant root system architecture. From these, fine lateral roots arise (secondary and tertiary roots) which actively explore the soil for water and nutrient capture, making up the majority of the length and root surface area (Hund *et al.*, 2009a). Changes in root system architectural traits and morphology have been shown to impact largely on cereal crops productivity in different water-limited environments (Comas *et al.*, 2013; Lynch *et al.,* 2014).

Deep rooting has been widely recognised as a key target to increase water uptake from deeper soil layers and improve yield in water scarce environments (Slafer *et al.*, 2005; Araus *et al.*, 2008; Tuberosa, 2012; Comas *et al.*, 2013). This phenotype allows for a sustained stomata opening when water is scarce in the top soil layers, favouring crop productivity (Hund *et al.*, 2009a). Wheat (Lopes and Reynolds, 2010) and rice (Kato *et al.*, 2006; Steele *et al.*, 2013) deep-rooted genotypes have shown yield advantage under drought, and the genetic dissection of this complex trait has revealed its large contribution to yield stability in water scarce environments (Uga *et al.,* 2011; Uga *et al.*, 2013). Genotypic variation for root morphological traits like root growth angle, root diameter and rate of elongation could define the ability of accessing water from deep soil layers (Araki *et al.*, 2002; Kato *et al.*, 2006; Chapagain *et al.,* 2014). Also, anatomical modifications such as the development of root cortical aerenchyma (i.e. the conversion of living cortical tissue into air space) reduces the metabolic costs of root soil exploration which has been related to increased root length density deeper in the soil profile and greater water uptake in maize exposed to drought (Zhu *et al.,* 2010).

In addition, an extensive and vigorous root system (e.g. large root biomass and increased root length density) has also been found advantageous for mitigating drought effects in different cereal crops (Hund *et al.*, 2009a; Chloupek *et al.*, 2010; Naz *et al.*, 2012), particularly in areas subjected to variations in seasonal rainfall patterns such as the Mediterranean environments. Conversely, vigorous roots may quickly deplete soil moisture in environments relying on stored soil water, which could risk crop production substantially (Palta *et al.*, 2011). In this type of environment, roots systems with a reduced seminal root xylem vessel diameter have been found advantageous for maintaining steady water use through crop development and optimise water use efficiency for improved yield under drought (Richards and Passioura, 1989).

Other root features such as greater diameter and root hairs have been found to favour root soil penetration in hard drying soils. Thicker roots show increased bending stiffness which facilitates root penetration through strong soil layers (Clark *et al.*, 2008). Improved root penetration ability becomes especially important at the beginning of drought stress, when small decreases in soil water content can lead to large reductions in the soil matric potential and so greater soil mechanical impedance (Bengough *et al.*, 2011). In this situation, increased soil strength may become the major constraint for root growth (Cairns *et al.*, 2004). Genotypic variation for root diameter has been associated with differences in root penetration ability and the capacity to better stand drought in the field (Steele *et al.*, 2013). Additionally, root hairs are a fundamental trait for anchoring roots in the soil and enabling root elongation into high-strength soils (Haling *et al.*, 2013). Moreover, root hairs are essential binding components of the rhizosheaths which are fundamental for ameliorating drought effects and nutrient acquisition under stress (Brown *et al.*, 2012). This root anatomical trait has been less explored for improving crop performance although initial results appear promising since it can be easily measured and significant genotypic variation has been found for the trait (George *et al.*, 2014).

1.3 Barley molecular breeding

The advent of molecular marker technology in the 1980s constituted an outstanding breakthrough for achieving crop improvements more efficiently than with conventional breeding approaches based upon assessment of plant phenotypes (Bernardo, 2008). The identification of DNA polymorphisms and development of genotyping methodologies allowed the integration of genotypic with phenotypic data and loci linked to specific traits to be identified. Molecular breeding studies started focusing on economically important traits such as yield, yield quality, disease resistance and more recently, abiotic stress tolerance, for which genetic dissection could provide the means for crop improvement through marker assisted selection (MAS) programmes. These traits show continuous variation and their performance is controlled by multiple genes or quantitative trait loci (QTLs) whose expression may be modulated by environmental factors (Falconer and Mackay, 1996). The development of robust statistical tools together with the continuous improvement of molecular marker technologies and effective phenotyping methods has been necessary for establishing quality marker–trait associations in QTL mapping studies and target genomic regions to be deployed in MAS programmes (Holland, 2007; Xu and Crouch, 2008). The advances achieved in molecular breeding during the last decades referred to barley and the prospects for future breeding programmes are discussed below.

1.3.1 Barley as a model crop for genetic studies

Among cereals, barley is considered as a good model plant to explore the genetics underlying complex traits, especially those related to abiotic stress tolerance given its broad geographic

adaptability (Ellis *et al.*, 2002; Tondelli *et al.*, 2006). Barley's diploid nature (2n= 2x= 14) makes genetic studies simpler to perform than for other polyploid members of the Triticeae tribe such as the hexaploid wheat (*Triticum aestivum*). Although self-fertilisation is predominant in barley, the ease of cross hybridisation between plants and subsequent inbreeding enables traits to be fixed in a population within a few generations facilitating the development of mapping populations for QTL identification.

Compared to other model species, the barley genome is large. It spans about 5.1 Gigabases (Gb) contained within seven pairs of chromosomes, approximately twelve times that of rice (430 Mb in ten pairs of chromosomes, IRGSP (2005)), with at least 80% of highly repetitive DNA (IBSC, 2012).

1.3.2 Advances in molecular markers and genetic maps

In the last thirty years, the progress in genotyping technology has progressively contributed to the understanding of genome organisation, genotypic variation and species phylogeny. In the beginning, morphological (i.e. visual characteristics such as growth habit) and biochemical markers (i.e. isozymes) were successfully used to investigate some traits of economic importance in crops (Eagles *et al.*, 2001). However due to their limited number and large variability they were rapidly superseded by molecular markers (i.e. DNA based marker systems).

1.3.2.1 DNA- based molecular markers

Hybridisation-based markers such as the Restriction Fragment Length Polymorphism (RFLP) are performed by hybridizing a chemically labelled DNA probe to a Southern blot of DNA digested with a restriction endonuclease. These assays are very reliable and enabled the establishment of one of the first barley DNA markers genetic map (Graner *et al.*, 1991); however this technology is expensive, and labour intensive which limits sample throughput significantly.

Polymerase Chain Reaction (PCR)-based markers took over rapidly, and DNA molecular markers such as Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and microsatellite or Simple Sequence Repeats (SSR) became important tools in barley genetics research, particularly SSRs and AFLPs since RAPDs were shown to be unreproducible (Collard *et al.*, 2005).

Amplified Fragment Length Polymorphism, or AFLP markers, are largely reliable and efficient compared to RFLPs and RAPDs. The technique is based on the selective PCR amplification of restriction fragments from digested genomic DNA, which yields many DNA polymorphisms in a single reaction. AFLPs allowed some of the first genetic studies on barley origin and domestication (Badr *et al.*, 2000; Komatsuda *et al.*, 2004) as well as genetic diversity studies

using different groups of germplasm (Russell *et al.*, 1997; Waugh *et al.*, 1997). In addition, these DNA-fingerprinting assays enabled the establishment of whole-genome genetic maps (Hori *et al.*, 2003). However, the main drawback from a breeding perspective is that these are dominant markers and could not be easily transferred to crosses with different parents (Graner *et al.,*2011).

SSRs markers (also called microsatellites), in contrast, are better suited for breeding. These consist of simple tandem repeated short units of 2–6 base pairs in length which are amplified by PCR using pairs of oligonucleotide primers specific to unique DNA sequences flanking the SSR sequence (Kashi *et al.,* 1997). SSR motifs are abundant in the barley genome and provide greater polymorphism than RFLPs, RAPD or AFLPs (Russell *et al.*, 1997). Also, SSRs are co-dominantly inherited, show even distribution throughout the genome and can be transferred among diverse crosses, which makes them suitable for marker-assisted selection programmes (Gupta and Varshney, 2000; Graner *et al.,* 2011). In barley, SSRs have been used in genetic diversity studies (Russell *et al.*, 2000; Ivandic *et al.*, 2002) and also in the establishment of a second-generation linkage map (Ramsay *et al.*, 2000), which has been effectively used in the identification of candidate target loci for crop improvement (Ellis *et al.*, 2002; Matus *et al.*, 2003; Pillen *et al.,*2003). However these are limited because of the time and effort needed to design and develop the primers (Collard *et al.*, 2005).

Today, these molecular markers have been surpassed in favour of new marker technologies providing reliable high-throughput genotypic information such as Single Nucleotide Polymorphisms (SNPs).

SNP genotyping

Sequence data quickly positioned SNP polymorphisms as the most common form of DNA variation occurring in both coding and non-coding regions of the genome (Rafalski, 2002a). Within the coding regions, the variation of a single base may change amino acids (nonsynonymous SNPs) which could affect the function of proteins, resulting in altered phenotypes. Synonymous SNPs do not alter the amino acid sequence and therefore are neutral, although they could generate a potential splice site that may result in phenotypic changes (Comadran *et al.*, 2012). Hence, SNP markers are a very valuable tool to mine new genetic variation to be exploited for crop improvement not only because of their high-throughput but also because of their potential to identify causal variants for a particular trait (Bedada *et al.*, 2014).

The nearly unlimited supply of SNP markers and the increased throughput of genotyping technologies have enabled the implementation of cost-effective genotyping technologies that have made SNP genotyping platforms the preferred methodology in plant breeding. Either a single line can be genotyped with 1000s of SNPs in a single reaction, e.g. Barley Illumina

iSelect 9K SNP chip (Comadran *et al.*, 2012) (7842 polymorphic SNPs), or a single SNP polymorphism can be characterised in a large group of lines, e.g. KASP marker system (LGC Genomics).

1.3.2.2 Genetic linkage maps

With the advances in the detection of DNA based molecular markers, the construction of genetic linkage maps is providing the framework to map simple and complex traits effectively (Collard *et al.*, 2005). Linkage maps describe the relative order of genes or genetic markers in linkage groups based on the recombination events that occurred during the development of a reference mapping population (i.e. advanced backcross population, double haploids, F_2 or recombinant inbred lines). The distance between markers is expressed in units of recombination or centiMorgan (cM) and the linkage groups correspond to those loci occurring on the same chromosome. The greater the distance between two markers, the greater the chance of recombination occurring during meiosis (Van Ooijen and Jansen, 2013).

The continuous progress in genotyping technologies has increased the density of barley reference genetic maps. While the first RFLP and SSR barley linkage maps accounted for 30–40 recombination events per chromosome (Graner *et al.*, 1991; Ramsay *et al.*, 2000), today the most recent SNP high-density genetic map comprises 350–800 DNA polymorphisms per chromosome, a marker every 0.25 cM on average (Comadran *et al.*, 2012). Also, the unique marker segregation pattern of individual linkage maps has been combined in consensus maps that increase marker density considerably. The most recent high-density SNP consensus map for barley has 5665 SNP markers (580–1100 per chromosome) spanning a total length of 1113 cM and mapped into 2032 unique consensus positions or bins (Muñoz-Amatriaín *et al.*, 2014). Several consensus linkage maps have been drawn before to allow a tighter marker-trait association in genome wide association studies and facilitate the identification of markers for marker-assisted breeding. The majority of these have combined the information from different types of molecular markers (Rostoks *et al.*, 2005; Wenzl *et al.*, 2006; Marcel *et al.*, 2007), however high-density SNP consensus maps provide a more accurate reference for association genetic analysis. In addition, the increased number of markers (higher resolution) and improved marker order offers a more uniform platform to integrate genetic and physical maps (Close *et al.*, 2009; Muñoz-Amatriaín *et al.*, 2011, 2014).

1.3.3 Integration of genetic and physical maps

In order to accelerate the isolation of genes underlying phenotypic traits, the location of a target QTL region needs to be considered in relation to the physical map before setting up a MAS programme (Thomas, 2003). Markers closely linked to a QTL can be used to anchor the genetic to the physical map and identify the genes co-segregating with the QTL. Detailed sequence information for the target region can be exploited to identify candidate genes either by looking

at gene annotations (Ni *et al.*, 2009) or considering the conserved synteny between species to identify putative orthologs (Mayer *et al.*, 2011; Comadran *et al.*, 2012). However, it should be noted that the relationship between genetic and physical distance varies along the chromosome and a large physical distance may separate the marker from the target gene in the centromeric and pericentromeric regions, where recombination is restricted (Baker *et al.*, 2014). In this context, high-density genetic maps of gene-based markers are crucial for linking genetic and physical maps (Szűcs *et al.*, 2009).

1.3.3.1 Barley whole genome sequencing

In the last few years, significant progress has been achieved towards sequencing and assembling the barley genome (IBSC 2012; Mascher *et al.*, 2013a). The combination of map-based sequencing approaches and whole genome shotgun (WGS) sequencing have allowed about 80% (4 Gb) of the large barley genome which represents more than 90% of expressed genes to be assembled (IBSC, 2012). Briefly, a physical genome scaffold was outlined through sequential alignment of >570,000 Bacterial Artificial Chromosome Sequences (BAC) clones from six libraries of the North American six-rowed cultivar Morex (Schulte *et al.*, 2011). Then, the transcribed regions of the genome were annotated by RNA sequence data generated from eight different barley growth stages and full length cDNA libraries from the two-row malting cultivar Haruna Nijo (Matsumoto *et al.*, 2011) with 95% of the total transcript clusters (79379) anchored to the genome scaffold. Transcript sequence homology compared against other plant model genome sequences (i.e. rice, *Sorghum*, *Brachypodium* and *Arabidopsis*) was determined. This allowed about 33% of the transcribed loci as high-confidence genes (those with high level of homology with at least one reference genome) and 67% as low-confidence genes (lack of homology) to be defined (IBSC, 2012). Continuous efforts from the International Barley Genome Sequencing Consortium to unveil the complex barley genome sequence have recently achieved significant improvements towards barley high-quality reference genome (IBSC 2016 submitted).

1.3.4 Quantitative genetics approaches to mine natural genetic variation

1.3.4.1 Quantitative traits and QTL mapping

Unravelling the multigenic control underlying the variation of quantitative traits is the major challenge that breeders and geneticists face to increase crop productivity effectively through molecular breeding approaches (Luo *et al.,* 2002; Hayes *et al.*, 2003). As stated before, the continuous variation of economically important characters such as yield, yield quality, biotic and abiotic stress tolerance generally respond to the combined and additive effect of several genes which can be modulated by the environment. Identifying the chromosomal regions or quantitative trait loci (QTLs), and ultimately the genes, associated with these traits is the main focus of genetic or QTL mapping studies (Falconer and Mackay, 1996).

i. Linkage and association mapping studies

Traditionally, QTL mapping studies have investigated the co-segregation of molecular markers and phenotypes within the progeny of controlled bi-parental crosses (linkage mapping). However, advances in genomics, phenotyping technologies and statistical methods have also motivated QTL studies in natural populations or association panels of unrelated lines. These two approaches differ essentially on the control that the experimenter has over the recombination events that have occurred in the population (Myles *et al.*, 2009).

Linkage mapping restricts the localisation of QTLs to the meiosis or recombination breakpoints that occurred during the establishment of the population, giving a coarse QTL chromosomal position in the regions of 10–30 cM. The QTLs located are specific for the segregating population and their effects may be overestimated when the population is less than 150–200 lines (Chen, Chang and Anyia, 2011). Linkage mapping studies in barley experimental populations have been used effectively to dissect the genetic base of complex traits such as flowering time (Laurie *et al.*, 1995; Cuesta-Marcos *et al.*, 2008) and plant height (Bezant *et al.*, 1996) as well as yield and yield adaptability (Tondelli *et al.*, 2013; Mansour *et al.*, 2014), drought tolerance (Chen *et al.*, 2010) and disease resistance (Hofmann *et al.*, 2013). In addition, advanced backcrossed (AB) populations from wide crosses have improved the identification of favourable allelic variants from exotic germplasm for disease resistance (Yun *et al.*, 2006), nutrient and water stress tolerance (Sayed *et al.*, 2012; Schnaithmann and Pillen, 2012) and improved yield quality (Schmalenbach and Pillen, 2009). Additionally, near isogenic lines (NILs) derived from biparental populations facilitate the "mendelisation" of the quantitative trait, allowing the fine mapping of the QTL which provides the means for positional cloning (van Berloo *et al.*, 2001; Schmalenbach *et al.*, 2011).

Conversely, **association mapping (or linkage disequilibrium mapping)** explores the recombination events that have occurred in the heterogenic genetic background of an association panel of lines and QTLs are located within a few centimorgans. The strength of this approach relies on the fact that it uses a wide range of the natural genetic diversity for a trait, considering more than two allelic variants. This is a cost-effective QTL analysis in which only QTLs tightly linked to the marker will be located on the basis of linkage disequilibrium (LD), i.e. the non-random co-inheritance of alleles at proximate loci (Salvi and Tuberosa, 2005; Myles *et al.*, 2009). High-density high-resolution genetic linkage maps have promoted this sort of analysis (Waugh *et al.*, 2009; Pasam *et al.*, 2012). However, some artefacts such as population substructure need to be modelled to reduce the likelihood of detection of positive and false negative marker trait associations (Comadran *et al.*, 2011; Wang *et al.*, 2012).

In barley, the combination of existing phenotypic variety trial data and genotyping within a linkage disequilibrium approach has been shown to be highly cost-effective for assessing the

broad genetic variability for specific traits. QTLs for yield and its components have been mapped in selected collections of germplasm representative of the diverse geographical range (Kraakman *et al.*, 2004; Pasam *et al.*, 2012). Also, association panels including lines representing a wide spectrum of landraces and wild accessions have been used for linkage disequilibrium mapping studies aimed at locating genomic regions influencing the response of yield and its component to water deficit (Comadran *et al.*, 2008; Comadran *et al.*, 2011a; Varshney *et al.*, 2012) and disease resistance (Roy *et al.*, 2010). Many of the major associations found in these studies have been validated with results from linkage mapping studies and candidate genes have been directly identified using the conserved synteny approach (Cockram *et al.*, 2010; Wang *et al.*, 2012). However, small effect QTLs generally escape detection through this approach because it is difficult to detect rare alleles with high confidence unless their effect on the phenotype is very large (Nordborg and Tavaré, 2002; Rafalski, 2010; Comadran *et al.*, 2011b; Korte and Farlow, 2013). In this context, linkage mapping and association mapping have been regarded as complementary approaches for QTL mapping and the identification of causative genes. Donor parents for a marker-assisted backcross programme could be selected from association mapping scans to validate and further dissect target QTLs in the segregating progeny of a biparental cross, where indeed novel QTLs with small effect are more likely to be detected (Salvi and Tuberosa, 2005; Fukuoka, Nonoue and Yano, 2010; Rafalski, 2010).

ii. Advanced backcross populations

Wide crosses between elite and wild barleys followed by several backcrosses to the elite parent (BC_2 or BC_3 generations) allow the reduction of the frequency of deleterious wild parent donor alleles while preserving most of the agronomic characteristics of the crop in the derived introgression lines. This advanced backcross (AB) strategy combined with QTL analysis was suggested by Tanksley and Nelson (1996) to identify and transfer valuable alleles from unadapted lines (landraces and wild accessions) into established inbred lines. The suitability of this approach was initially reported for tomato (Tanksley *et al.*, 1996) and rice (Xiao *et al.*, 1996), and soon extended to other economically important crops such as maize (Li *et al.*, 2014) and wheat (Narasimhamoorthy *et al.*, 2006). A systematic research flow for accessing and dissecting the naturally occurring genetic variation for crop improvement through recurrent backcross strategy is well illustrated by Fukuoka *et al.* (2010) based on rice mapping populations (Fig. 1.7).

The development of Chromosome substitution lines through recurrent backcrosses in a MAS scheme facilitates the rapid progress in linkage mapping and validation of QTLs since it effectively bridges the coarse QTL detection at early generations with the validation and targeting of loci where exotic alleles may contribute genetic gains to the crop. In addition, rare alleles with a positive effect are more likely to be detected compared to other biparental populations such as F_2 and recombinant inbred lines (RILs). However, the detection of spurious associations may be greater (Fukuoka *et al.,* 2010). Several loci have been identified as good candidates to be introduced in plant breeding programmes by means of chromosome substitution lines; however, to date, only a handful of examples are found in the literature where backcrossing programmes have contributed to the development of new cultivars using crop wild relatives (Hajjar and Hodgkin, 2007; Feuillet *et al.,* 2008).

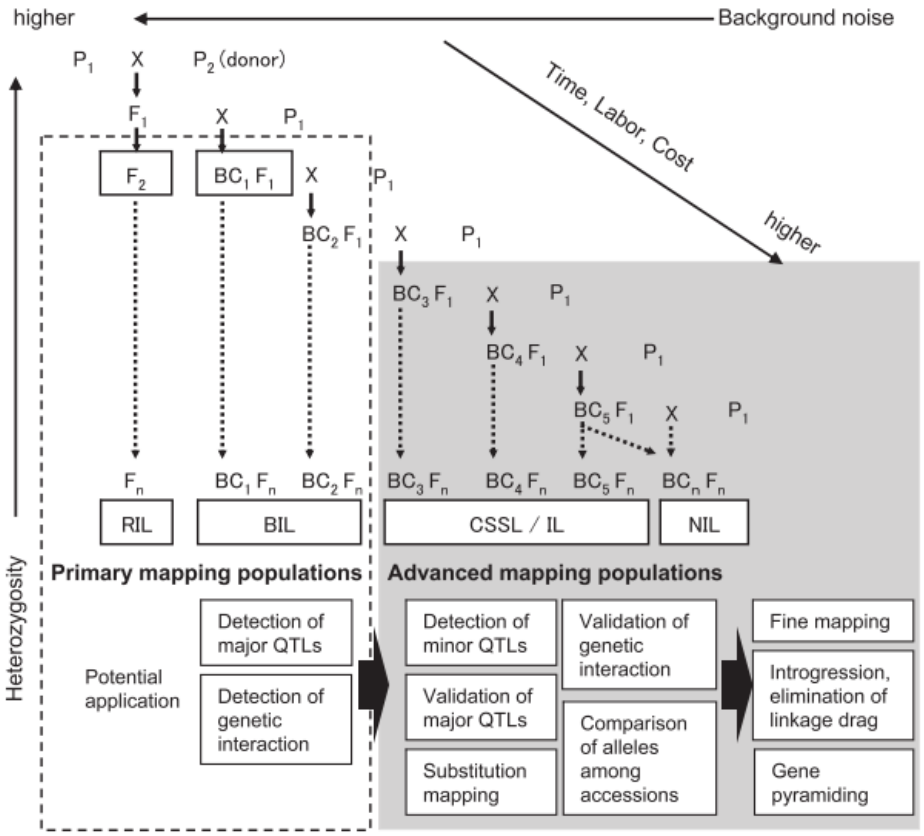

Figure 1.7 Research strategy for accessing the naturally occurring genetic variation and determining its utility for crop improvement using elite genetic backgrounds in rice biparental mapping populations: recombinant inbred line (RIL), backcrossed inbred line (BIL), chromosome segment substitution line (CSSL), introgression line (IL) and near-isogenic line (NIL).

(Adapted from: *S Fukuoka, Y Nonoue and M Masahiro, Breed Sci (2010) 60: 509-517*)

Barley AB-populations

Since Pillen *et al.,* (2003) and Matus *et al.*, (2003) reported the first advanced backcross populations for QTL mapping using spring barley, a few other sets of introgression lines have been developed and used in QTL studies (Table 1.1). These populations were established as 136 BC_2F_2 lines from the cross cv. Apex × ISR101-23 and 137 BC_2F_6 lines, from the cross cv. Harrington × Caesarea 26-24. Generally, elite spring two-rowed malting barleys have been selected as the recipient genetic background for recurrent crosses with wild barley accessions from the Fertile Crescent. Along with the population developed by Matus *et al.*, (2003), the North American cultivar Harrington was also used as recipient parent of a BC_2F_8 (n=98) population in which the wild accession OUH602 was the donor parent (Yun *et al.*, 2006). The natural resistance to foliar diseases observed previously in a set of Harrington/OUH602 RILs (Yun *et al.*, 2005) was verified and refined using this AB-population, which was also found to be effective for detecting novel unadapted alleles for improved crop performance (Gyenis *et al.*, 2007). The same wild accession (OUH602) was used as donor genome in crosses with the Japanese cultivar Haruna Nijo (Hori *et al.*, 2005). The authors developed BC_3F_2 (n=134) recombinant lines together with BC_3DH (n=93) population derived to validate QTLs found across these groups of lines. In this case the lines were evaluated for domestication and agronomic traits. This population was the first one genetically characterised with a high-density SNP platform showing the potential of recombinant chromosome substitution lines to derive QTL-fine mapping and QTL cloning studies (Sato and Takeda, 2009; Schmalenbach *et al.*, 2011). In addition, the selection of a minimum set of substitution lines representing the exotic genome by single marker-defined wild introgression in the uniform genetic background of the elite parent was also favoured by the increased accuracy of genetic maps. These "exotic introgression libraries" have been defined for the main AB-populations since they are considered permanent genetic resources that facilitate rapid screening of favourable traits in wild genomes (Zamir, 2001).

The barley AB-population obtained from the cross between the German elite malting barley cv. Scarlett and the wild accession from Israel "ISR42-8" has been the most studied (von Korff *et al.*, 2004). The favourable effect of the exotic genome at loci associated with disease resistance (von Korff *et al.*, 2005) agronomic performance (von Korff *et al.*, 2006) and malting quality (von Korff *et al.*, 2007) was initially revealed in studies conducted with 301 BC_2DH lines derived from the initial cross. Likewise, the wild barley genome was found to improve performance of morphological (i.e. root morphology) and physiological traits (i.e. proline accumulation) contributing plant adaptation to droughted environments (Sayed, 2011; Sayed *et al.*, 2012). Some of these results were validated using smaller groups of 39, 59 or 72 introgression lines obtained from subsequent backcrosses ($BC_3S_{4:6}$) and selected to represent

Table 1.1. Barley AB-QTL mapping studies.

Backcross population and number of lines (Hv xHsp)[1]	Molecular Markers	No QTLs[2]					Hsp (%)[3]	Reference
		A/M/P	M	R	Dr	Dm		
cv Harrington × Caesarea 26-24								
140 BC_2F_6	47 SSRs	17	16			4	<30	(Matus *et al.*, 2003)
80 BC_2F_6	47 SSRs	21					24	(Inostroza *et al.*, 2009)
80 BC_2F_6	765 SNPs	61					20	(GCP, 2010)
cv Apex × ISR101-23								
136 BC_2F_2	45 SSRs	86					34	(Pillen *et al.*, 2003)
cv Harry × ISR101-23								
164 BC_2F_2	45 SSRs	108					48	(Pillen *et al.*, 2004)
cv Barke × HOR11508								
123 BC_1F_2 (DH)	5 AFLPs, 100 SSRs	80					52	(Talamè *et al.*, 2004)
cv Brenda × HS213								
181 BC_3DH	400 SSRs	19	6				48	(Li *et al.*, 2005)
cv Haruna Nijo × H602								
134 BC_3F_2	25 SSRs, 60 EST	14				4	61	(Hori *et al.*, 2005)
cv. Harrington × H602								
98 BC_2F_8	111 SSRs				10		-	(Yun *et al.*, 2006)
98 BC_2F_8	113 SSRs	30					50	(Gyenis *et al.*, 2007)
cv Scarlett × IRS 42-8								
301 BC_2DH	98 SSRs				18		61	(von Korff *et al.*, 2005)
301 BC_2DH	98 SSRs	86					36	(von Korff *et al.*, 2006)
301 BC_2DH	98 SSRs		48				37.5	(von Korff *et al.*, 2007)
39 BC_3S_6	98 SSRs				15		33	(Schmalenbach *et al.,*, 2008)
39 BC_3S_6	98 SSRs	47					40.4	(Schmalenbach *et al.*, 2009b)
39 BC_3S_6	98 SSRs		40				15	(Schmalenbach and Pillen, 2009)
301 BC_2DH	106SSRs, 255DArT	55		15			34.1	(Sayed, 2011)
301 BC_2DH	106SSRs, 255DArT	8					37.5	(Sayed *et al.*, 2012)
28 BC_3S_4	98SSRs, 636 SNPs	65					35.4	(Schnaithmann and Pillen, 2012)
47 BC_3S_4	636 SNPs	44					-	(Honsdorf *et al.*, 2014a)
52 BC_3S_4	4201 SNPs	54					38.8	(Honsdorf *et al.*, 2014b)
72 BC_3S_6	636 SNPs			28			53.6	(Naz *et al.*, 2014)
301 BC_2DH	371 DArT+SSRs, 636 SNPs			33			45.5	(Arifuzzaman *et al.*, 2014)

[1] Population cross between an elite barley cultivar (*Hv*) used as recurrent parent and a wild accession (*Hsp*) as the donor parent of the cross.

[2] Number of QTLs located for agronomic, morphological and physiological traits (A/M/P), Malting traits (M), root traits (R), disease resistance (Dr) and domestication traits (Dm).

[3] % of loci where the wild barley alleles contributed positively on the trait performance.

most of the wild barley genome by single marker-defined introgressions in each line (Schmalenbach *et al.,* 2008; Schmalenbach and Pillen, 2009; Naz *et al.*, 2014). These studies have aided the identification of candidate genes underlying the QTLs and also target suitable introgression lines for map-based cloning and QTL pyramiding projects to enrich the crop genetic base with favourable exotic alleles.

1.3.4.2 cv. Harrington × Caesarea 26-24 RCSLs population

The development of the Recombinant Chromosome Substitution Lines population (RCSLs) derived from the cross between cv. Harrington and the wild barley accession Caesarea 26-24 (Matus *et al.*, 2003) was conducted according to the advanced backcross strategy proposed by (Tanksley and Nelson, 1996) (Fig. 1.8). The population was established as a BC_2F_6 population (137 lines) for which there was no conscious selection during generations. The authors selected the donor parent of the population, Caesarea 26-24, based on its passport data and the genetic distance from the elite cv. Harrington: the North American spring two-row malting quality standard used as the recurrent parent of the population. Once the population was established, the genotypic architecture of the lines was inferred using 47 SSR markers.

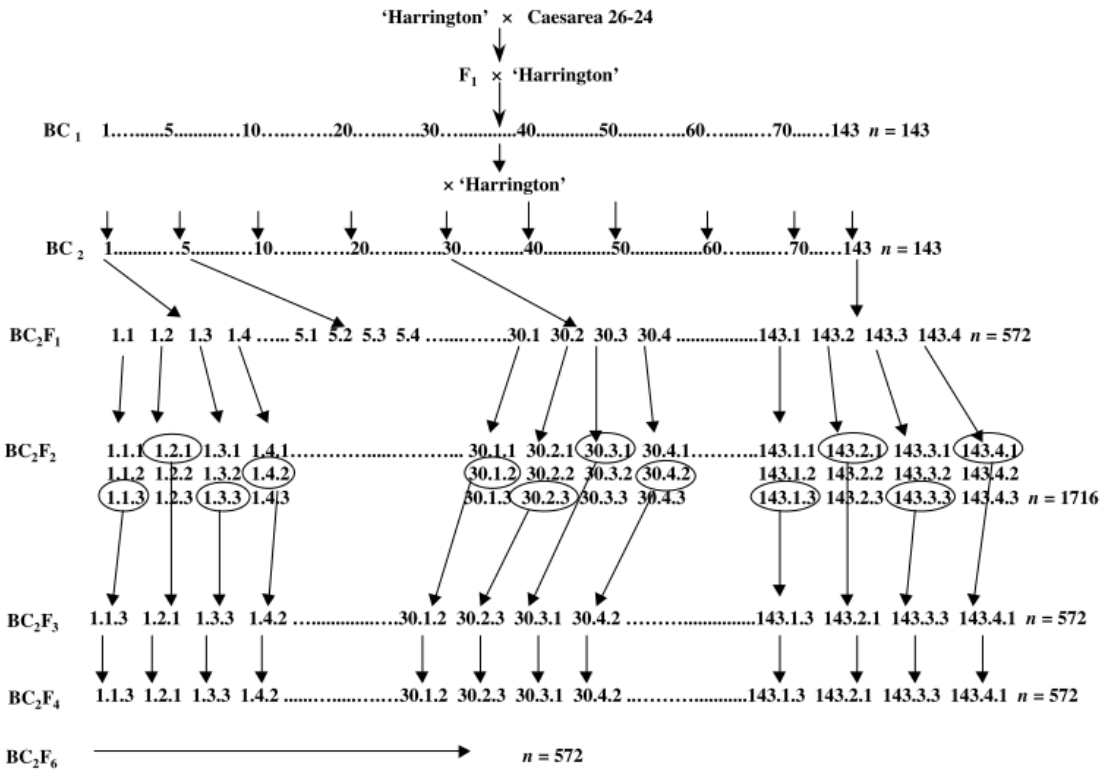

Figure 1.8 Advanced backcross strategy for the development and generation of the Recombinant Chromosome Substitution Lines (RCSLs) obtained from introgression of the wild barley accession Caesarea 26-24 (donor parent) into the elite genetic background of cv. Harrington (recurrent parent). Four seeds of one randomly selected plant from each set of three BC_2F_2 plants were planted to obtain the BC_2F_3 and advance the generation until BC_2F_6. One plant from each set of F_6 RCSLs, from there three plants were discarded for efficiency purposes, making up a final population of 137 BC_2F_6 lines that were genotypes with 47 SSRs.

Adapted from: *I Matus, A Corey, T Filichkin et al., Genome (2003) 46: 1010-1023*

i. Parents of the population

The elite cultivar Harrington (derived from the cross Klages/3/Gazelle/Betzes//Centennial) was bred at the Crop Science Department of the University of Saskatchewan (Canada) (Harvey and Rossnagel, 1984). For more than twenty years after its release in 1981, cv. Harrington was the primary two-row variety exploited commercially in Canada and the United States due to its outstanding yield and malt quality.

This elite cultivar has also been used for QTL mapping studies in the development of experimental biparental populations with other distinct malt cultivars such as cv. Morex (Marquez-Cedillo *et al.*, 2000) and in other wide crosses with wild barley accessions (Yun *et al.*, 2006; Roy *et al.*, 2010). The former were focused on detecting malting quality QTLs that could improve the crop malting properties whereas the latter was aimed at dissecting the genetic basis of leaf diseases to which cv. Harrington is susceptible.

The genetically distant wild barley accession Caesarea 26-24 (also named as OSU11 and Hvs11) was collected from a dry and high salt area in Israel, presumably from the site of origin with the same name (Latitude 32.50°N, Longitude 34.9°E) as described in Pakniyat *et al.*, (1997). This accession has been used in studies exploring the natural genetic diversity existing in the wild barley genepool for tolerance to drought and salt stress (Pakniyat *et al.*, 1997; Ellis *et al.*, 2000) and also in the flowering time component studies and the molecular characterisation of the vernalisation genes governing its phenotype (Karsai *et al.*, 2004; von Zitzewitz *et al.*, 2005).

ii. Previous studies

The RCSLs have been phenotypically characterised for agronomic and malting traits (Matus *et al.*, 2003), yield and drought related traits (Inostroza *et al.*, 2007, 2009; GCP, 2010; del Pozo *et al.*, 2012) and other characteristics that may be involved in an enhanced drought tolerant phenotype such as seedling vigour (Inostroza *et al.*, 2011) and the stem soluble carbohydrate content (Mendez *et al.*, 2011). In all studies, the variability in response to drought stress in the RCSL population suggests that *H. vulgare* subsp. *spontaneum* has contributed alleles that increased drought tolerance in some of the genotypes. The field evaluations were conducted in different growing season and Mediterranean environments in Chile differing in water availability: Cauquenes under dryland condition and Santa Rosa under irrigated condition. Also two locations in Oregon over the 2004 growing season were considered in a preliminary association analysis for drought related traits with 47 SSR markers (Inostroza *et al.*, 2009). Even though the genome coverage was limited, 21 chromosome associations were identify for the agronomic traits studied, with 24% of the exotic alleles contributing favourable alleles.

For the Generation Challenge Programme (2010, unpublished) the genetic architecture of the RCSLs was re-established with the 1536-SNP multiplex assay (Close *et al.*, 2009). The information gathered for 765 polymorphic SNPs allowed the introgression of *H. vulgare* subsp. *spontaneum* alleles to be refined and a new QTL mapping study conducted. In this case data from three Chilean environments in the 2009 growing season was used. Target regions of the wild donor genome that confer increased drought tolerance were identified. Twenty percent of the alleles that were reported for the dry sites of Santa Rosa and Cauquenes were found to have positive effects from the introgressed wild barley donor. Finally, based on the genotypic data a minimum set of 29 RCSLs was selected to constitute the "exotic introgression library" to represent the genome of Caesarea 26-24 and conduct QTL fine mapping studies. This group of RCSLs is the main focus of study of the present project.

1.4 Research objectives

Whilst improving yield and yield quality has been the major objective in barley breeding programmes, the need for more tolerant and resilient cultivars has also become an important target to mitigate the effect that unpredictable changing environments have in crop production. In this regard, the natural genetic diversity associated with the wild barley genepool constitutes a rich reservoir of novel alleles that could broaden the genetic base of the cultivated form substantially, particularly for coping with abiotic stresses such as drought. In addition, the role of root systems and their variability is becoming a new and promising breeding target for enhancing crops water productivity. However, exotic genetic diversity remains unexploited and more effective approaches are required for achieving further genetic gains using unadapted germplasm while maintaining crop high productivity.

Advanced backcrossed mapping populations such as the RCSLs developed by Matus *et al.*, (2003) are an effective approach to genetically dissect some of the naturally occurring variation that could be utilised in crop breeding programmes. In addition, selected minimum sets of lines or introgression libraries representing the genome of the wild donor parent of these populations have been proposed as "reagents" to optimise the screening for multiple phenotypes and discover genes that underlie traits of agricultural value.

In this context, and in light of previous studies showing the potential of the RCSLs as a valuable source of new allelic variation for improved crop performance in water scarce environments, a set of 29 RCSLs representing the genome of the exotic parent of the population have been investigated further with the following objectives:

1. Evaluate the effect of the exotic donor genome in the performance of the elite recurrent parent and identify morphological, agronomical and developmental aspects that might contribute increased adaptability of the crop to water deficit in field conditions (Chapter 2).
2. Assess the genetic control of quantitative traits measured in the field trials using a suitable marker–trait association analysis designed for this minimum group of lines. Identify loci and causative genes governing the phenotypic variation observed in the field and target those that would be useful to future research in sets of near isogenic lines (NILs) (Chapter 3).
3. Explore the phenotypic variation in the RCSLs root system using a simple experimental approach to infer whether the exotic genome contributes variation in root architectural traits that could be important in the crops adaptability to water scarce environments (Chapter 4).

2 RCSLs phenotypic characterisation in field conditions

Abstract

Twenty-nine barley Recombinant Chromosome Substitution Lines (RCSLs) derived from a cross between an elite barley cultivar 'Harrington' used as the recurrent parent, and *Hordeum vulgare* subsp. *spontaneum* accession from a very dry region in the Fertile Crescent, as the donor parent (Matus *et al.,* 2003) were used to investigate the effect of the exotic genome introgressions from the wild barley in the performance of the elite variety and test whether it contributes favourable alleles improving drought tolerance.

Field trials were conducted under rain-out shelters to evaluate the effect of water stress in the RCSLs performance during 2013 and 2014 growing seasons. Morphological, developmental and agronomic traits showed consistent and wide variation over both years. A mixed model analysis and an additive-main-effects-multiplicative interaction (AMMI) model were optimised to study the phenotypic variation observed and characterise the lines according to their similarities or dissimilarities with the elite parent, as well as for stability in their phenotype across environments.

Despite the fact that the exotic introgressions did not improve yield under drought in the cultivated barley, some RCSLs showed significantly improved performance compared to cv. Harrington in terms of grain quality. For example, OSU040 was shown to be remarkably superior for TGW regardless of the water treatment. In addition, genotypes such as OSU060 and OSU053 were able to maintain high yield potential values while accounting for broader adaptability than the cultivated barley for seed quality.

The data generated from these experiments establishes the basis for defining the chromosome regions involved in the RCSLs phenotype (Chapter 3) and the identification of contrasting lines to investigate the contribution of root traits in the phenotypic variation observed (Chapter 4).

2.1 Introduction

Breeding programmes have been very successful increasing yield potential and quality of main cereal crops such as barley. The newly released cultivars are generally well adapted to high-input environments, and perform reasonably well under water-limiting conditions (Cattivelli *et al.*, 2008). However, despite the fact that yield and yield components can still be significantly improved within the elite germplasm, in some cases this improvement seems to be less marked or has reached a plateau (Thomas, 2003). At the same time, the selection process has limited the genetic variability for improving adaptation to abiotic stresses (Tanksley and McCouch, 1997; Forster *et al.*, 2000). For this reason, locally adapted barley varieties and wild accessions have become a very valuable genepool to explore new allelic variation towards the development of improved and broadly adapted cultivars (Ellis *et al.*, 2000; Forster *et al.*, 2000; Newton *et al.*, 2011).

Since wild and domesticated barley are self-pollinated and interfertile, it is possible to tap into the genetic diversity of the exotic unadapted germplasm and test its potential for enriching the genetic basis of the crop by developing advanced backcrossed populations such as the barley Recombinant Chromosome Substitution Lines (RCSLs) developed by Matus *et al*. (2003). Previous studies have highlighted the potential of this group of lines to investigate the drought tolerance responses in barley as they show a wide genetic variability for this trait under Mediterranean conditions (Inostroza *et al.*, 2007; del Pozo *et al.*, 2012). It seems that the combination of different traits might influence the level of response to water deficit in the RCSLs (Inostroza *et al.*, 2011; Mendez *et al.*, 2011). In addition, some chromosomal regions have been correlated with favourable effects on agronomic and malting quality traits (Matus *et al.*, 2003; Inostroza *et al.*, 2009). All these studies have been carried out using different sets of RCSLs selected from the 137 lines that constitute the original population (Matus *et al.*, 2003). For the present study, a set of 29 RCSLs and cv. Harrington have been selected to investigate their performance under water deficit in the field.

However, phenotyping for drought tolerance is a challenging task, not only because of the variation of the drought adaptation responses and the multigenic nature of the trait, but also because of the difficulties in defining the intensity of the stress which ultimately will lead to different levels of response (Araus *et al.*, 2002; Passioura, 2007; Tuberosa, 2012).

Glasshouse experiments in pots are widely used to study drought tolerance in crop plants. This approach allows water treatments to be accurately defined under controlled environmental conditions, making the results obtained easier to interpret and more repeatable. However, there are some important disadvantages and generally, the outcomes from these studies are difficult to extrapolate to the field conditions (Passioura, 2006b; Poorter *et al.*, 2012). Although,

phenotyping for drought in field trials brings associated experimental "noise" as a consequence of uncontrolled environmental conditions, field based approaches are more realistic and relevant from a crop breeding perspective (Passioura, 2007, 2012; Cattivelli *et al.*, 2008). Therefore, in order to understand the effects of water deficit on plants phenotyped in the field it becomes essential to characterize the environmental conditions as to the water status of the soil and the climatic conditions in which plants are being evaluated (Jones, 2007).

Equally important is choosing an effective statistical analysis able to test the variation in the phenotype observed not only due to genotypic and the environmental variation, but also to the interaction of these two sources of variation (GE interaction). The fluctuations in yield performance influenced by the environmental conditions are responsible for the differences between yield potential and actual yield. Selection of high-yielding genotypes with an improved stability across target environments is an important goal for breeding programmes to minimize this 'yield gap', especially when environmental constraints define less-favourable conditions for crop development (Teulat *et al.*, 2001; De Vita *et al.*, 2010). In addition, it is important to elucidate the role of secondary traits on crop performance, especially under stress conditions as they may be particularly suited for improving selection response to stress conditions (Araus *et al.*, 2008)

Chapter 2 focuses on the study of the phenotypic variation observed in 29 RCSLs evaluated under different water treatments in a "semi-control" field trial at The James Hutton Institute, Dundee. The main objective of this experiment was to define the effect of the exotic genome in the performance of the elite variety (cv. Harrington) as well as identify morphological and developmental traits that might contribute to enhance the agronomic performance of the crop under less favourable conditions.

2.2 Material and Methods

2.2.1 Plant material

Matus *et al.* (2003) developed a set of barley Recombinant Chromosome Substitution Lines (RCSLs) aiming to access unexploited wild barley allelic variability for barley crop improvement (Fig. 2.1). 140 RCSLs were obtained through an advanced backcross strategy (Tanksley and Nelson, 1996) between an elite north American spring malting barley 'Harrington' used as the recurrent parent and *Hordeum vulgare* subsp. *spontaneum* accession (Caesarea 26-24) from a very dry region in Israel, as the donor parent (Matus *et al.*, 2003). The 137 RCSLs obtained after two backcrosses and six generations of selfing (BC_2F_6) were initially genotypically characterized with 47 mapped SSRs (Matus *et al.*, 2003) and more recently, as part of a Generation Challenge Programme (2010, unpublished data), with a 1536-SNP multiplex assay (Close *et al.*, 2009) using Illumina Golden Gate Bead Array technology (Fan *et al.*, 2006).

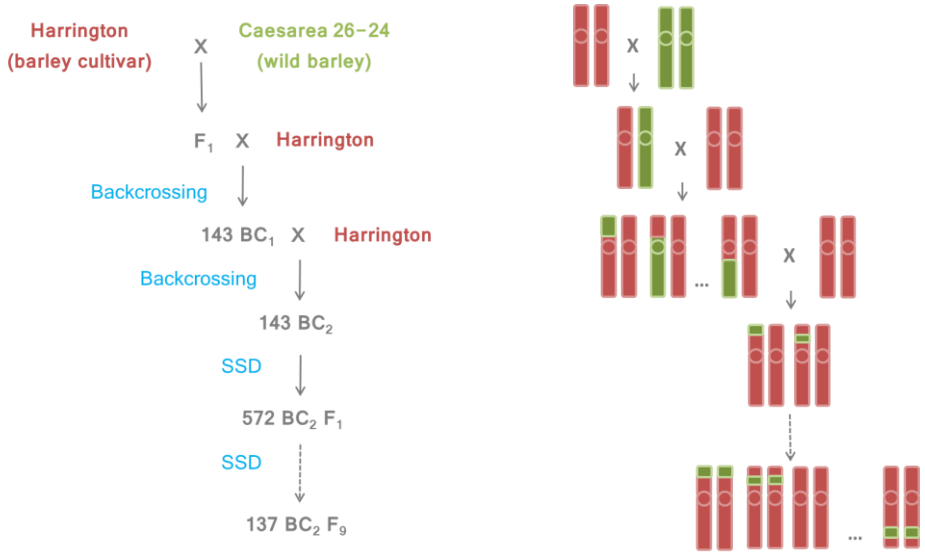

Figure 2.1. Development of the recombinant chromosome substitution lines through the advanced backcross strategy of Tanksley and Nelson (1996). The accession of *Hordeum vulgare* subsp. *spontaneum* (Caesarea 26-24, represented in green) was used as donor parent and the elite cultivated barley (cv. Harrington, represented in red) as the recurrent parent (Matus *et al.* 2003).

A group of 29 RCSLs was selected for the present study to assess the effect of wild barley introgressed segments in the elite barley genetic background and its contribution to the crop

performance under water deficit conditions. The selection was based on the genotypic architecture of the lines and a minimum tilling panel for each of the barley chromosomes using data generated as part of the GCP project 2010 (unpublished data). This group of lines represents the entire exotic genome through overlapping chromosome introgressions of the *Hordeum vulgare* subsp. *spontaneum* accession (Close *et al.* 2009; GCP project 2010 unpublished data). In Chapter 3, more detailed genotypic information obtained as part of this study for the 29 RCSLs and cv. Harrington using the 9 K Infinium SNP iSelect platform (Comadran *et al.*, 2012) will be described.

2.2.2 Field experiment

2.2.2.1 Field site and experimental layout

The 29 RCSLs and the recurrent parent, cv. Harrington, were grown in field trials in 2013 and 2014 growing seasons at The James Hutton Institute (Latitude 56.45°N, Longitude 3.06°W). Each plot consisted of six 0.8 m long rows with a gap of 0.2 m between them. Plots within the same column were separated by 0.23 m and the distance between each of the five columns established was 0.3 m. The experiment was designed with CycDesigN (VSN International, UK) as a row column design with the treatment and replicates superimposed on top (Fig. 2.2, Appendix 1). Four replications and three water treatments were established. Two rows of guard plots (cv. Concerto) were sown between the replicates within each water treatment and along the sides of the trial to minimize the edge effect. In addition, an extra row of guard plots was sown at the opened ends of the rain-out shelter and along the sides of the field trial to reduce the amount of water coming from rainfall (Fig. 2.3). Two gaps around 3 m were left between water treatments.

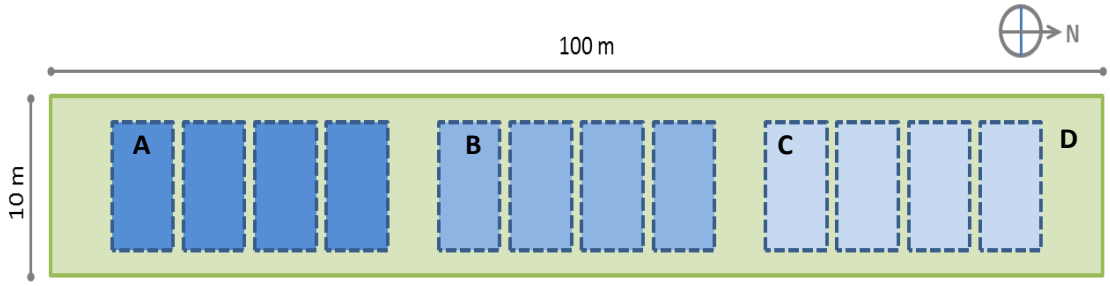

Figure 2.2 Field experimental design. Row column design with three water treatments superimposed on top and four replicates within each treatment. A) Full irrigation B) Partial irrigation C) Drought (no irrigation) D) Guard plots

Sowing dates were 15th and 17th April in 2013 and 2014 respectively. Seeds were sown using a Wintersteiger Seedmatic drill at 4 cm depth. In 2013 the granulated fertilizer HYDRO Sulphur cut (22% N, 4% P_2O_5, 14% K_2O, and 7.5% SO_3) was used as a seed dressing and in 2014 as a top-dress fertilizer. The fertilizer was applied at 270 kg ha^{-1} the day of sowing and up to 500 kg ha^{-1} a week after sowing following the local agronomic practices for spring barley. To minimize the effect of pests, field trials were treated with fungicides Bravo 500 (1 l ha^{-1}), $Siltra_{XPro}$ (0.6 l ha^{-1}), Vegos (0.25 l ha^{-1}) and Justice (0.15 l ha^{-1}) the 7th and 9th June and for aphids on 11th and 17th July in 2013 and 2014 respectively.

The rain-out shelter was built up and skinned with polythene film 0.15 mm thick Clear-High UV (Visqueen, UK) around a month after sowing: the 16th May 2013 and 14th May 2014. At this stage, seedlings were already established in the field at growth stages GS13–GS14 (Zadoks *et al.*, 1974). There was an average of 15 plants per row within a plot. The sprinkler irrigation system was set up in the irrigated plots a few days after the rain-out shelter was established, defining the different water treatments from the 23rd May in 2013 and 21st May in 2014 (Fig. 2.3).

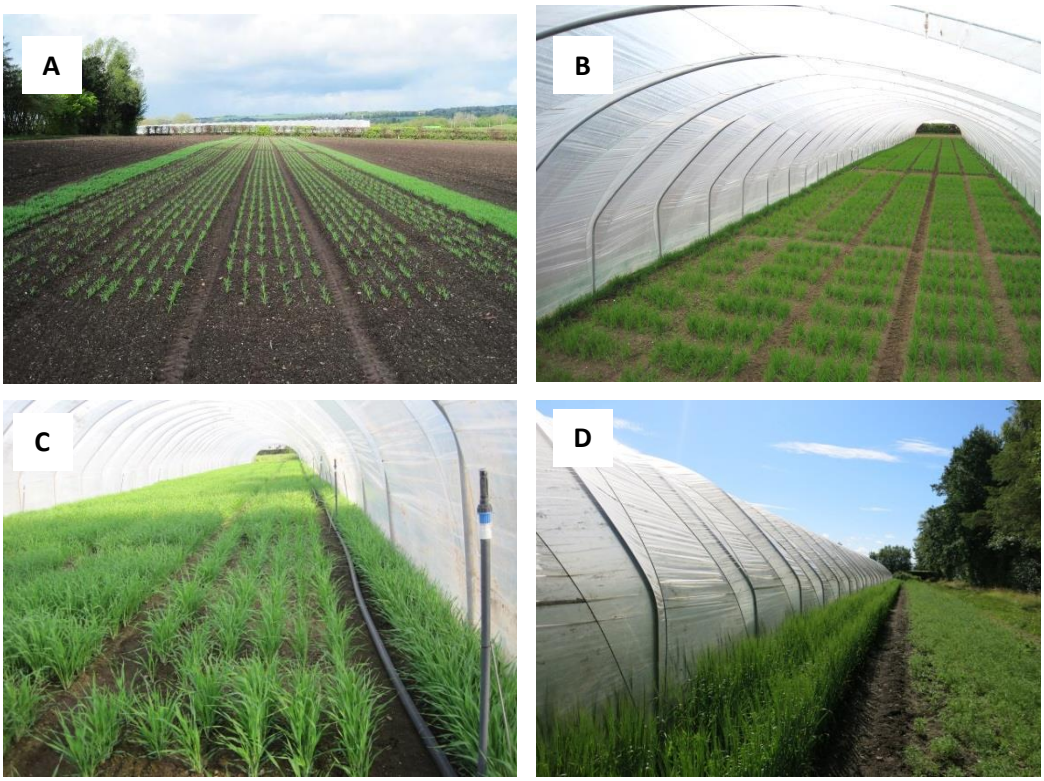

Figure 2.3. Field experiment layout A) 22 day-old seedlings at growth stage GS13 a few days before establishing the rain-out shelter and the irrigation system in the 2014 field trial. B) A row column design was established with the replicates and treatments superimposed on top; view of two of the water treatments. C) Sprinkler irrigation system for the irrigated plots. D) Guard plots along the sides of the rain-out shelter.

2.2.2.2 Water treatments

Three water treatments were established to assess the response to water deficit on the development of the crop: two irrigated treatments and a non-irrigated one. The irrigated plots were watered based on the Met Office rainfall average records for east Scotland during the barley growing season (http://www.metoffice.gov.uk/climate). A sprinkler irrigation system was used to water the plots twice a day with a total volume of 2.5 mm per day. The irrigation was stopped at different growth stages in these two treatments: at grain milk development (GS 73–77) in fully irrigated plots (23rd July in 2013 and the 24th July in 2014) and before anthesis (GS 50–55) in the partially irrigated plots (24th June in 2013 and 23rd June in 2014). Therefore, the total accumulated irrigation in the partially irrigated plots was 80 mm in 2013 and 82.5 mm in 2014 and in the irrigated plots it was 155 mm and 162.5 mm respectively (Table 2.1). The non-irrigated plots constituted the drought treatment and they were not watered after the rain-out shelter was built.

The soil moisture content in the field was monitored on a weekly basis by measuring it with a capacitance probe PR2/4 (Delta-T Services Ltd) at different depths within the soil profile in 53 access tubes evenly distributed across the field trial. The PR2/4 contains electronic sensors at 10, 20, 30 and 40 cm depth which transmit an electromagnetic field into the soil surrounding the access tubes. The HH2 Moisture meter readout unit (Delta-T Services Ltd) connected to the probe converts the output signal voltage returned in mV into soil moisture content (%).

Meteorological data of the 2013 and 2014 growing seasons were obtained from The James Hutton Institute weather station records (Latitude 56.45°N, Longitude 3.07°W). Daily air and soil temperature and rainfall accumulated were considered to define the climate conditions prior to the setup of the rain-out shelter and throughout the field experiments.

Table 2.1 Water treatments summary table. Accumulated irrigation (mm) for each water treatment and duration of the irrigation referred to the growth stages of the crop.

	Full irrigation	**Partial Irrigation**	**Drought**
Irrigation accumulated	155 mm in 2013 163 mm in 2014	80 mm in 2013 83 mm in 2014	No irrigation
Growth stages[1]	GS 13–GS 75	GS 13–GS 55	n/a

[1] Growth stages according to Zadoks *et al.* (1974)

2.2.2.3 Soil physical characteristics

Soil matric potential and soil strength were measured for intact field cores (40 mm height, 55 mm diameter) sampled ten days after sowing in the 2013 field trial. The samples were obtained using a manual auger at three different depths (0–5, 20–25, 40–45 cm) in six representative locations throughout the field trial. Water retention of these cores was assessed by applying nine different suctions ranging from 0 to 1500 kPa. The water content of the samples was calculated when the cores reached stable weight. Suctions 0.5 kPa, 1 kPa, 2 kPa, 5 kPa, 10 kPa, 25 kPa and 50 kPa were applied using tension tables formed from a semipermeable membrane (EchoTech, Bonn, Germany). For 250 and 1500 kPa suction, a pressure plate was used (ELE International, Hemel Hempstead, UK). For the 1500 kPa measure, small core samples (10 mm height, 20 mm diameter) were taken from the initial ones. These data were used to define the field soil water characteristics curve using the software RETC (van Genutchen *et al.*, 1991).

In addition, the field soil mechanical impedance was determined to estimate whether a decrease in matric potential in the field soil due to the water stress could have restricted root growth. The penetrometer resistance was measured on samples equilibrated at suctions 10 kPa, 25 kPa, 50 kPa and 250 kPa using a 2 kN universal mechanical test machine (Model 5544, Instron, High Wycombe, UK) and a needle penetrometer of 0.91 mm diameter with a penetration rate of 4 mm min^{-1} and a depth of probe of 15 mm was used. Bluehill2 software (Instron, High Wycombe, UK) was used to analyse the data.

2.2.2.4 Phenotypic analysis

Morphological, developmental and agronomic traits were measured in the 2013 and 2014 field experiments (Table 2.2). Yield and yield components data were assessed by harvesting and threshing the two middle rows of each experimental plot when plants had reached physiological maturity. The plants were hand harvested and mechanically threshed using a stationary Wintersteiger KG laboratory thresher (F. Walter and H. Wintersteiger, Ried/Innkreis, Austria). Calculations were done based on the Yield and Yield components practical guide, CIMMYT (2013), using the weight of seeds harvested from 0.4 m^2 per plot, dried at 100°C for 24 hours. The average weight of 1000 grains (TGW) and seed morphological traits were obtained from samples of dried seeds using a Marvin Digital Seed Analyser (GTA Sensorik GmbH, Germany). Additionally, presence or absence of qualitative traits such as glossy spike, seed shattering, grain threshability, purple grain and waterlogging was scored for the different genotypes. Spikes harvested from the remaining rows were threshed and processed for future seed stocks.

Table 2.2. Phenotypic traits registered in the 2013 and 2014 field trials.

Trait	Abbreviation	Units	Method of measurement
Morphological traits			
Total Height[1]	HEI	cm	Plant height from soil surface to the tip of the ear (excluding awns)
Collar Height[1]	COL	cm	Plant height from soil surface to the main stem collar
Peduncle length[1]	PdL	cm	Distance measured in a main stem from the first node to the collar
Peduncle extrusion[1]	PdE	cm	Distance measured in a main stem from the flag leaf to the collar
Ear length[1]	EAR	cm	Length of the ear excluding awns
Seed Area	SdA	mm^2	Average area of seed determined by image analysis using MARVIN grain analyser
Seed Length	SdL	mm	Average length of seed determined by image analysis using MARVIN grain analyser
Seed Width	SdW	mm	Average width of seed determined by image analysis using MARVIN grain analyser
Developmental traits			
No. tillers	TILL	tillers	Number of tillers harvested from the two middle rows of each plot
Heading date	HEA	DAS[2]	Number of days from sowing to half of ear emergence (GS55)
Agronomic traits[3]			
Dry Yield	DY	$kg.ha^{-1}$	Weight of grain collected from two middle rows of each plot calculated for an hectare
Thousand Grain Weight	TGW	$g.1000\ grains^{-1}$	Average weight of 1000 grains determined by image analysis using MARVIN grain analyser
Harvest Index	HI	%	Ratio of dry yield to above ground dry biomass for the two middle rows of each plot calculated for an hectare
Biomass Yield	BY	$kg.ha^{-1}$	Above ground dry biomass obtained from two middle rows of each plot
Qualitative traits			
Glossy spike	GLS		Presence / absence of glossy spikes
Seed shattering	SHT		Presence / absence of seed shattering
Grain threshability	THR		Presence / absence awn retention
Lodging	LOD		Presence / absence of lodging

[1] Heights were measured in three main stems from the two middle rows of each plot at harvest maturity (GS 92)
[2] DAS: Days after sowing
[3] Agronomic traits based on the two middle rows harvested from each plot and samples dried for 24h at 100^oC

2.2.3 Data analysis

2.2.3.1 Analysis of variance of phenotypic data

Statistical analyses for testing the effect of the water treatments on the performance of the different lines across years were carried out using GenStat 17th Edition (VSN International, UK). Residual maximum likelihood method (REML) was used to estimate fixed effects and random effect parameters in the traits measured (Payne *et al.*, 2011). Thus, a three factorial mixed model analysis was used to test the effect of the year, the water treatment, the genotype and their interaction in the performance of each trait and statistical significance for the fixed model effects was assessed by using a chi-squared based Wald-test. The random term included the replicate and the column to take into account the spatial variation in the field trials. The model was defined as follows (Appendix 2):

Equation 1

$$X_{ijklmn} = \mu + G_i + T_j + G_i * T_j + Y_k + G_i * Y_k + T_j * Y_k + R_l(T_j * Y_k) + C_m(T_j * Y_k * R_j) + \varepsilon_{n(ijklm)}$$

where X_{ijklmn} is the phenotypic performance of a trait, μ is the general mean, G_i is the fixed effect of the *i*-th genotype, T_j is the fixed effect of the *j*-th water treatment, G_i*T_j is the fixed effect of the interaction of the *i*-th genotype and the *j*-th treatment, Y_k is the fixed effect of the *k*-th year, G_i*Y_k is the fixed effect of the interaction of the *i*-th genotype and the *k*-th year, T_j*Y_k is the fixed effect of the interaction of the *j*-th treatment and the *k*-th year, $R_l(T_j*Y_k)$ is the random effect of the *l*-th replicate nested in *j*-th treatment and *k*-th year, $C_m(T_j*Y_k*R_l)$ is the random effect of the *m*-th column nested in the *j*-th treatment, *k*-th year and *l*-th replicate and $\varepsilon_{n(ijklm)}$ is the residual term of X_{ijklmn}.

Similarly, a two factorial mixed model was used when needed to assess the effect of the water treatment, the genotype and their interaction in the performance of the traits considering each field trial separately. In this case the model was defined as follows (Appendix 2):

Equation 2

$$X_{ijkl} = \mu + G_i + T_j + G_i * T_j + R_k(T_j) + C_l(T_j * R_k) + \varepsilon_{l(ijk)}$$

where X_{ijkl} is the phenotypic performance of a trait in one field trial, μ is the general mean, G_i is the fixed effect of the *i*-th genotype, T_j is the fixed effect of the *j*-th water treatment, G_i*T_j is the fixed effect of the interaction of the *i*-th genotype and the *j*-th treatment, $R_k(T_j)$ is the random effect of the *k*-th replicate nested in *j*-th treatment, $C_l(T_j*R_k)$ is the random effect of the *l*-th column nested in the *j*-th treatment and *k*-th replicate, and $\varepsilon_{l(ijk)}$ is the residual term of X_{ijkl}.

2.2.3.2 Line × phenotype associations

Following the mixed model analysis, a Fisher's least significance difference (LSD) test was used to compare the Best Linear Unbiased Estimates (BLUEs) of the fixed terms at 0.01 levels of probability. The BLUEs for cv. Harrington were used as a control to define groups of RCSLs with a significantly reduced or improved performance compared to the elite barley main performance or in its interaction with the water treatment. The VMCOMPARISON procedure in Genstat 17 was used for this analysis. Introgressed chromosomal regions from the wild barley present on groups of RCSLs showing a significant genotypic effect in the same direction might harbour the same target exotic alleles that could potentially be associated with the phenotype observed.

To determine the RCSLs with a consistent improved or diminished phenotype due to its genotype or in response to water stress, the comparison was performed using the BLUEs for the genotypes as well as at the genotype × treatment interaction level.

2.2.3.3 Phenotypic correlation between the traits investigated

Correlation analysis was conducted using the correlation function in Genstat 17 in order to define the relationship between secondary traits and yield under optimum irrigation and under stress for the experimental conditions. Therefore, phenotypic correlations between plant developmental, morphological and agronomic traits were determined using the Pearson's correlation coefficient (r). Comparisons were made also for the same trait in contrasting water regimes.

2.2.3.4 Impact of drought on yield

In order to determine whether the introgressed chromosome regions of the exotic genome enhance the performance of the cv. Harrington under water deficit conditions without compromising its high yielding capability the following analyses were performed.

i. Drought tolerance index (DTI)

To determine the impact of drought in crop productivity for each genotype, the drought tolerance index (DTI) was calculated considering 2013 and 2014 field together. DTI was calculated based on the Drought Sensitivity Index (DSI) proposed by Fischer and Maurer (1978). However, DTI used the fraction of yield conserved during drought whereas DSI used the fraction of yield that is lost during drought:

Equation 3

$$DTI = \frac{DY\ ws / DY\ ww}{\overline{DY}\ ws / \overline{DY}\ ww}$$

In other words, DTI measures the ratio of yield maintained under water stress (DY ws) compared to the potential yield or yield under well-watered conditions (DY ww) of a genotype in relation to the Drought Intensity Index (DII) or mean fraction of yield conserved across all genotypes under drought ($\overline{DY}$ ws) compared to the overall yield potential ($\overline{DY}$ ww). DII, the denominator for calculating DTI, is understood as an environmental stress intensity index which measures the general effect of drought on crop production over all genotypes.

Genotypes with a DTI value of 1 or larger are considered as tolerant to drought for the experimental conditions, likewise genotypes with a DTI value smaller than 1 are considered as drought sensitive. Correlation between DTI and genotypic means for the different traits was investigated for each treatment.

ii. Yield components genotype by environment interaction

In order to assess the pattern of the RCSLs responses across different environments, the additive main effects and multiplicative interaction model (AMMI) was used. In this case six environments were defined as the combination of growing season and water treatment. Through this method, the overall variation observed for yield traits (dry yield and thousand grain weight) was partitioned into genotype main effects, environment main effects and genotype × environment (GE) interactions (Gauch, 2006). This model combines a principal component analysis (PCA) of the GE interaction that is obtained as a result of a two factorial analysis of variance taking genotype and environment as the main effect. As a result, the interaction principal components generated (IPCA1 and IPCA2) are used to graphically summarise (biplots) the GE variation observed. The first IPCAs generated account for most of the GE variation across testing environments and the scores of each genotype for each principal

component axis can be considered as dynamic stability parameters. The AMMI2 biplot uses the genotypes and environments scores for the first two IPCA components (IPCA1 on the X-axes and IPCA2 on the Y-axes) giving information about the GE patterns observed. In addition, the AMMI1 biplot tests the genotypes yield potential and stability simultaneously by plotting in the same diagram the average yields (X-axes) and the first dimension measure of GE interaction (IPCA1) for both genotypes and environments (Y-axes). AMMI test was conducted using Genstat 17.

2.3 Results

2.3.1 2013 and 2014 environmental conditions

2.3.1.1 General climatic data

Mean air and soil temperatures and values of accumulated rainfall registered in the weather station at The James Hutton Institute, Dundee, were used to characterize the meteorological conditions preceding the 2013 and 2014 growing seasons as well as throughout the duration of the field trials (Table 2.3 and Appendix 3).

The meteorological conditions prior to setting up the field trial in 2014 were milder compared to the 2013 conditions (Table 2.3 and Appendix 3). From January until April 2014 the mean air maximum temperature (9.17°C) and mean air minimum temperature (2.80°C) were higher than in 2013 with values of 6.03°C and 0.43°C respectively. The soil temperature at 10 cm depth was also considerably higher in 2014 than in 2013 (4.38°C and 2.15°C). In terms of accumulated rainfall for the same period, 2013 had less rain (216.5 mm compared to 230.6 mm in 2014) corresponding to fewer rainy days (66 compared to 78 in 2014).

The seedling establishment in both field trials occurred under similar climatic conditions (Table 2.3 and Appendix 3). From sowing date until the rain-out shelter was built, air temperatures were slightly milder in 2014 than in 2013 (maximum of 14.4°C in 2013 and 15.3°C in 2014 and minimum of 5.5°C in 2013 and 7.2°C in 2014). This difference between years was greater for soil temperature at 10 cm depth. In 2014 this value ranged from 7.2°C to 12.8°C (10.4°C average value) whereas in 2013 the temperature ranged from 2.8°C to 11.4°C (7.9°C on average). These differences might have influenced the establishment of the seedlings as well as the vegetative growth of the plants throughout the season.

All plots reached heading in a shorter period of time in 2014 (11 days) than in 2013 (18 days) under slightly different conditions (Table 2.3 and Appendix 3). During this time, the minimum and maximum air temperatures outside the rain-out shelter were on average higher in 2014 (a minimum of 11.0°C and a maximum of 19.5°C) than in 2013 (8.93°C and 17.3°C). Although the temperature within the shelter was not recorded, it is assumed that the temperatures followed a similar trend.

Table 2.3. Climate data before sowing, during seedling establishment, before heading and during heading in the 2013 and 2014 growing seasons. Air and soil mean temperature values (± SE) and accumulated rainfall (mm) values obtained from the James Hutton Institute weather station (56.45°N; 3.07°W).

		Mean temperatures in ^{o}C (± SE)			
Time period *Year*	Date (No days)	Air Max (min–max)	Air min (min–max)	Soil ± SE (min–max)	Rainfall[1] (days)
Before sowing					
2013	01 Jan – 15 Apr	6.03 ± 0.26	0.43 ± 0.22	2.15 ± 0.16	216.5
	(105 days)	(1.60 – 13.90)	(-5.00 – 8.80)	(-1.20 – 7.20)	(66)
2014	01 Jan – 17 Apr	9.17 ± 0.26	2.80 ± 0.25	4.38 ± 0.19	230.6
	(107 days)	(4.70 – 15.70)	(-2.50 – 7.80)	(0.70 – 9.00)	(78)
Seedling establishment					
2013	16 Apr – 16 May	12.63 ± 0.46	4.63 ± 0.42	7.92 ± 0.32	63.7
	(31 days)	(3.80 – 17.70)	(1.60 – 8.70)	(2.80 – 11.40)	(18)
2014	18 Apr – 14 May	13.83 ± 0.43	5.79 ± 0.58	10.44 ± 0.30	60.3
	(27 days)	(9.80 – 17.20)	(-0.70 – 9.90)	(7.20 – 12.80)	(22)
Before heading					
2013	15 Apr – 07 Jun	14.42 ± 0.46	5.51 ± 0.38	9.82 ± 0.41	n/a
	(54 days)	(3.80 – 21.40)	(1.60 – 12.40)	(2.80 – 17.60)	n/a
2014	17 Apr – 06 Jun	15.31 ± 0.39	7.24 ± 0.44	12.01 ± 0.31	n/a
	(51 days)	(9.80 – 20.50)	(-0.70 – 12.80)	(7.20 – 16.10)	n/a
During heading					
2013	08 Jun – 25 Jun	17.27 ± 0.39	8.93 ± 0.48	15.02 ± 0.33	n/a
	(18 days)	(14.20 – 20.10)	(5.90 – 12.10)	(12.50 – 17.00)	n/a
2014	07 Jun – 17 Jun	19.56 ± 0.45	10.97 ± 0.47	15.93 ± 0.27	n/a
	(11 days)	(18.30 – 23.80)	(8.30 – 13.70)	(15.00 – 18.30)	n/a

2.3.1.2 Water treatments characterization

In order to define the differences between the water treatments, changes in the soil water content at 10, 20, 30 and 40 cm depth were monitored on a weekly basis for the three water treatments in each field trial (Fig. 2.4). Together with this, a simple characterization of the soil matric potential and soil strength was carried out using 18 soil cores sampled at three different depths (0–5, 20–25, 40–45 cm) in the field site the first year of experiments. This information was used to obtain water release curves for each depth and to infer the mechanical impedance for root elongation as the soil water potential decreased as a consequence of the water deficit in the field (Appendices 4 and 5).

At the beginning of the experiments the soil moisture was similar across the three water treatments. The highest values were registered at 30 cm depth, around 35%vol (=0.3 cm^3/cm^3)

for both years. From this point, there was a progressive decrease in moisture in the soil profile of the droughted plots, reaching the lowest values at 10 cm depth towards the end of the growing season in both trials (6%vol in 2013 – around 0.07 cm^3/cm^3 – and 7%vol – 0.08 cm^3/cm^3 – in 2014). The variation observed in the watered plots was in the same range until the irrigation was stopped in the partially irrigated plots (70 DAS in 2013 and 67 DAS in 2014). From this moment, the decrease in water content at the four different depths was noticeable in these plots, especially in 2013 when the soil moisture registered at the end of the season was comparable to the droughted plots one.

It is quite remarkable the low values of water content registered at 40 cm depth all through the duration of the 2013 experiment, not only in the droughted plots but also in the irrigated ones. From the beginning to the end of the season there was very little variation in soil moisture at this level (14%vol – 0.16 cm^3/cm^3 – to 12.3%vol – 0.14 cm^3/cm^3 – in the full irrigated, 13.6%vol – 0.16 cm^3/cm^3 – to 8.8%vol – 0.10 cm^3/cm^3 – in the partial irrigated and from 13.2%vol to 8.4%vol in the drought treatment). On the contrary, the water content at 40 cm in 2014 was considerably higher for all the water treatments with the lowest values registered in water deficit plots at the end of the season (12.6%vol) and values over 20%vol (=0.2 cm^3/cm^3) in the irrigated regimes. The reduction in matric potential as a consequence of the lack of water at 40 cm depth might have contributed to increase the mechanical impedance of the soil, being a limitation for root growth below 40 cm in the soil profile in 2013. In fact, the soil moisture values registered in 2013 at this depth correspond to estimated suction pressures much larger than 250 KPa. The penetrometer resistance estimated using soil samples collected at this depth and equilibrated at 250 KPa suction was 2.75±0.54 MPa (Appendices 4 and 5), average value higher than the 2MPa limit defined as significantly restrictive soil penetration resistance for root elongation in arable soils (Bengough *et al.*, 2011). Therefore, it is likely that the high mechanical impedance reached in the field soil at 40 cm depth caused a major limitation for root elongation below this limit in the 2013 field trial and thus, it limited the root soil exploration for deep soil moisture in the water deficit plots.

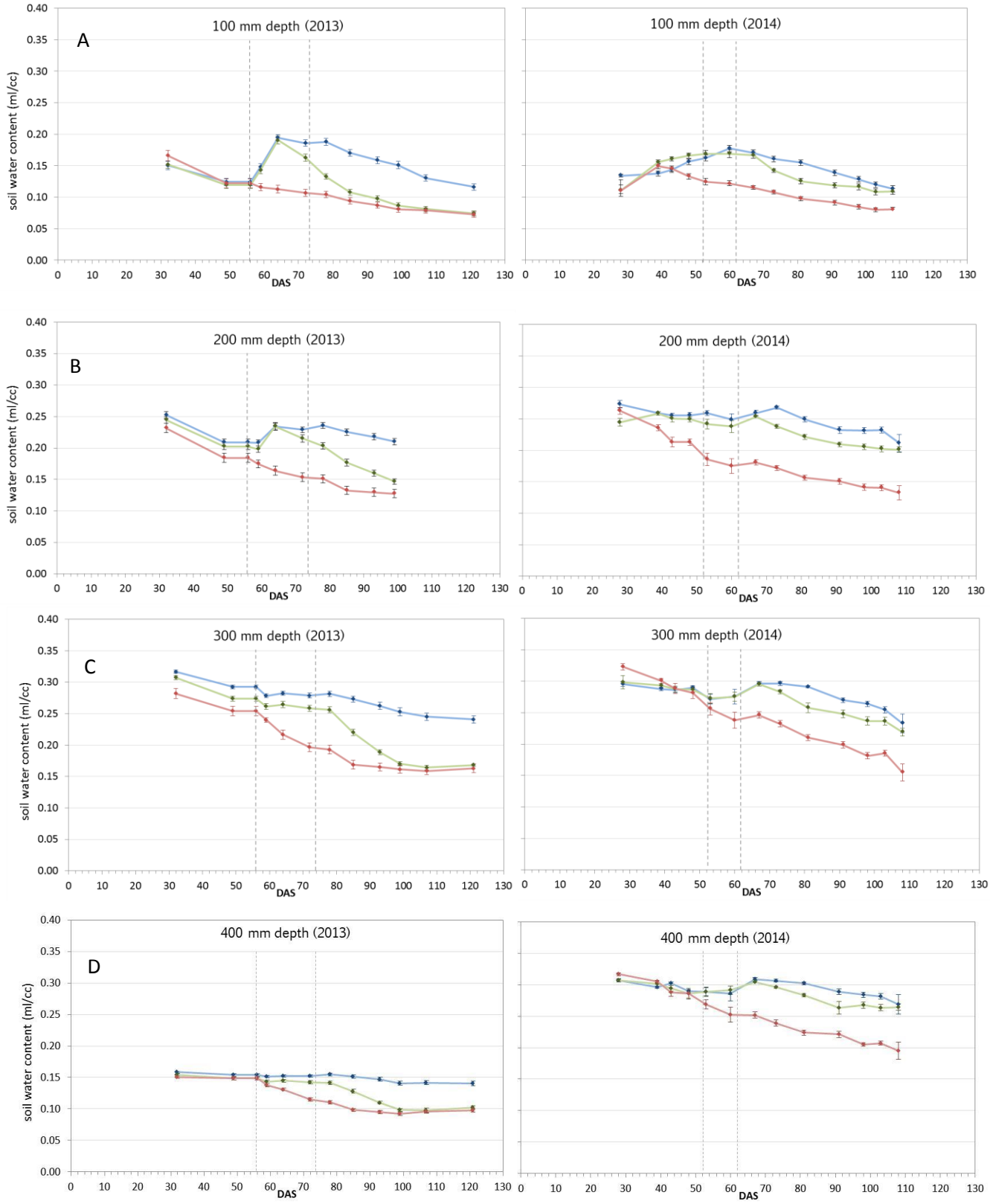

Figure 2.4. Volumetric water content (ml cm^{-3}) in the soil profile at (A) 100 mm, (B) 200 mm, (C) 300 mm and (D) 400 mm depth in the full irrigated (blue), partial irrigated (green), and drought (red) water treatment in 2013 and 2014 field trials. Vertical dashed lines indicate beginning and end of heading time referred to days after sowing (DAS) for year. Error bars indicate standard error of the mean.

2.3.2 RCSLs characterization under field conditions

2.3.2.1 Phenotypic characterization of the RCSLs and cv. Harrington in three water regimes

29 RCSLs and cv. Harrington were characterised for three water treatments (Full Irrigation, Partial Irrigation and Drought) and across two years field trials (2013, 2014) where the following morphological, developmental and agronomic traits were determined: total height (HEI), collar height (COL), peduncle length (PdL), peduncle extrusion (PdE), ear length (EAR), seed area (SdA), seed length (SdL), seed width (SdW), number of tillers (TILL), heading date (HEA), dry yield (DY), thousand grain weight (TGW), harvest index (HI) and biomass yield (BY). The analysis of variance of the phenotypic data showed highly significant differences ($P<0.01$ and $P<0.001$) among genotypes, treatments, years and their interaction for most of the traits measured (Tables 2.4, 2.5, 2.6).

For all traits, the effect of genotype was highly significant, suggesting the presence of wide genetic variability in the RCSLs. In addition to this, the significant interaction of genotype with treatment was high for most of the traits, indicating a range of response to the water treatments across the different genotypes and therefore, an interesting dataset to identify superior genotypes. In addition, a strong effect of the year, the water treatment and their interaction, was significant for the majority of the traits, suggesting not only a strong environmental effect on the different traits scored across the years but also differences in the environmental conditions across years that might have contributed to the intensity of the stress and therefore, the performance of the different lines. These differences were taken into account in order to investigate the impact of drought on the RCSLs agronomic performance as well as for the other traits. It is important to note that the results presented and discussed herein correspond to 28 RCSLs instead of 29 RCSLs since OSU016 had to be discarded for the analysis due to the ambiguous phenotypic and genotypic data gathered for this line (see section 3.3.1).

i. Morphological traits

Plant height

Significant variation in plant height was observed among the RCSLs essentially due to the genotype and the water treatment. Plants under drought were significantly shorter than plants in the irrigated plots (Fig. 2.5). The mean value for collar height under drought in 2013 was 94.6 cm and under full irrigation 105.4 cm, similar values were observed in 2014 (94.8 cm and 106.1 cm). The effect of year was insignificant and measured as a highly heritable trait.

In general, RCSLs were taller than Harrington with mean collar height values across the years of 102.5 cm and 91.7 cm respectively (data not shown). The RCSLs collar heights ranged from 78.0 cm to 137.7 cm under full irrigation in 2013 (Harrington between 91.3 cm to 94.0 cm). Similar observations were made in 2014 (Table 2.4).

Table 2.4. Morphological traits. Means (± SE) and maximum and minimum range values (in brackets) for collar height (COL), peduncle length (PdL), ear length (EAR), seed area (SdA) and seed length (SdL) for cv. Harrington (Harr) and the RCSLs under Full irrigation (FI), partial irrigation (PI) and drought (DR) conditions in two years field trials (2013 and 2014).

Trait / Treatment	2013		2014		Effect [1]
	Harr	RCSLs	Harr	RCSLs	
COL (cm)					
FI	93.2±0.6	105.8±1.0	96.2±1.7	106.5±0.9	Y ns
	(91.3–94.0)	(78.0–137.7)	(92.3–100.3)	(83.0–136.0)	T ***
PI	94.8±2.0	107.7±1.0	97.3±2.1	105.2±1.0	G ***
	(90.7–100.0)	(87.0–136.3)	(92.0–102.0)	(81.3–137.3)	Y × T ns
DR	83.4±1.0	95.0±0.9	85.3±0.5	95.2±0.8	Y × G ***
	(81.0–85.7)	(70.3–122.3)	(84.0–86.3)	(80.3–125.0)	T × G ***
					Y × T × G ns
PdL (cm)					
FI	31.4±0.7	38.6±0.4	26.3±1.6	32.9±0.4	Y ***
	(30.0–33.0)	(31.0–50.3)	(22.7–30.3)	(20.7–45.3)	T ***
PI	31.6±1.2	39.4±0.4	28.9±0.4	32.5±0.4	G ***
	(28.3–33.7)	(28.7–64.0)	(28.0–30.0)	(23.3–44.7)	Y × T *
DR	28.0±0.7	32.9±0.4	26.3±2.0	29.3±0.4	Y × G ***
	(27.0–30.0)	(24.0–43.7)	(22.0–30.0)	(20.7–40.3)	T × G **
					Y × T × G ns
EAR (cm)					
FI	8.8±0.3	9.3±0.1	8.2±0.4	8.9±0.1	Y ***
	(8.0–9.7)	(6.7–12.0)	(7.3–9.3)	(7.0–11.3)	T ***
PI	9.2±0.2	9.3±0.1	8.6±0.4	8.9±0.1	G ***
	(8.7–9.7)	(7.3–13.0)	(7.7–9.3)	(6.3–13.0)	Y × T ***
DR	7.7±0.3	8.7±0.1	8.3±0.4	8.8±0.1	Y × G ***
	(7.0–8.3)	(7.0–10.7)	(7.3–9.0)	(6.7–12.7)	T × G ns
					Y × T × G *
SdA (mm^2)					
FI	22.2±0.1	22.9±0.2	20.7±0.8	22.3±0.2	Y ***
	(21.9–22.4)	(19.6–29.3)	(19.6–22.9)	(19.0–29.1)	T *
PI	22.3±0.1	23.8±0.1	21.2±0.6	22.7±0.2	G ***
	(22.1–22.7)	(21.1–30.1)	(20.0–22.3)	(19.0–28.9)	Y × T ns
DR	22.8±0.2	24.2±0.1	21.2±0.7	22.3±0.1	Y × G ***
	(22.3–23.0)	(22.0–29.1)	(19.9–23.1)	(19.0–26.8)	T × G *
					Y × T × G ns

Table 2.4. Continued

Trait / Treatment	2013		2014		Effect [1]
	Harr	RCSLs	Harr	RCSLs	
SdL (mm)					
FI	7.8±0.0	8.2±0.1	8.0±0.2	8.5±0.1	Y ns
	(7.7–7.9)	(6.9–12.0)	(7.7–8.5)	(7.0–12.2)	T *
PI	8.1±0.0	8.7±0.1	8.0±0.2	8.5±0.1	G ***
	(8.0–8.2)	(7.6–12.7)	(7.6–8.3)	(7.1–11.7)	Y × T **
DR	8.1±0.0	8.7±0.0	7.8±0.1	8.3±0.1	Y × G *
	(8.0–8.1)	(8.0–10.9)	(7.6–8.1)	(7.3–11.2)	T × G **
					Y × T × G ns

[1] Statistically significant effect of the year (Y), water treatment (T), the genotype (G) and their interaction (YxT, YxG, TxG, YxTxG) on each trait performance. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

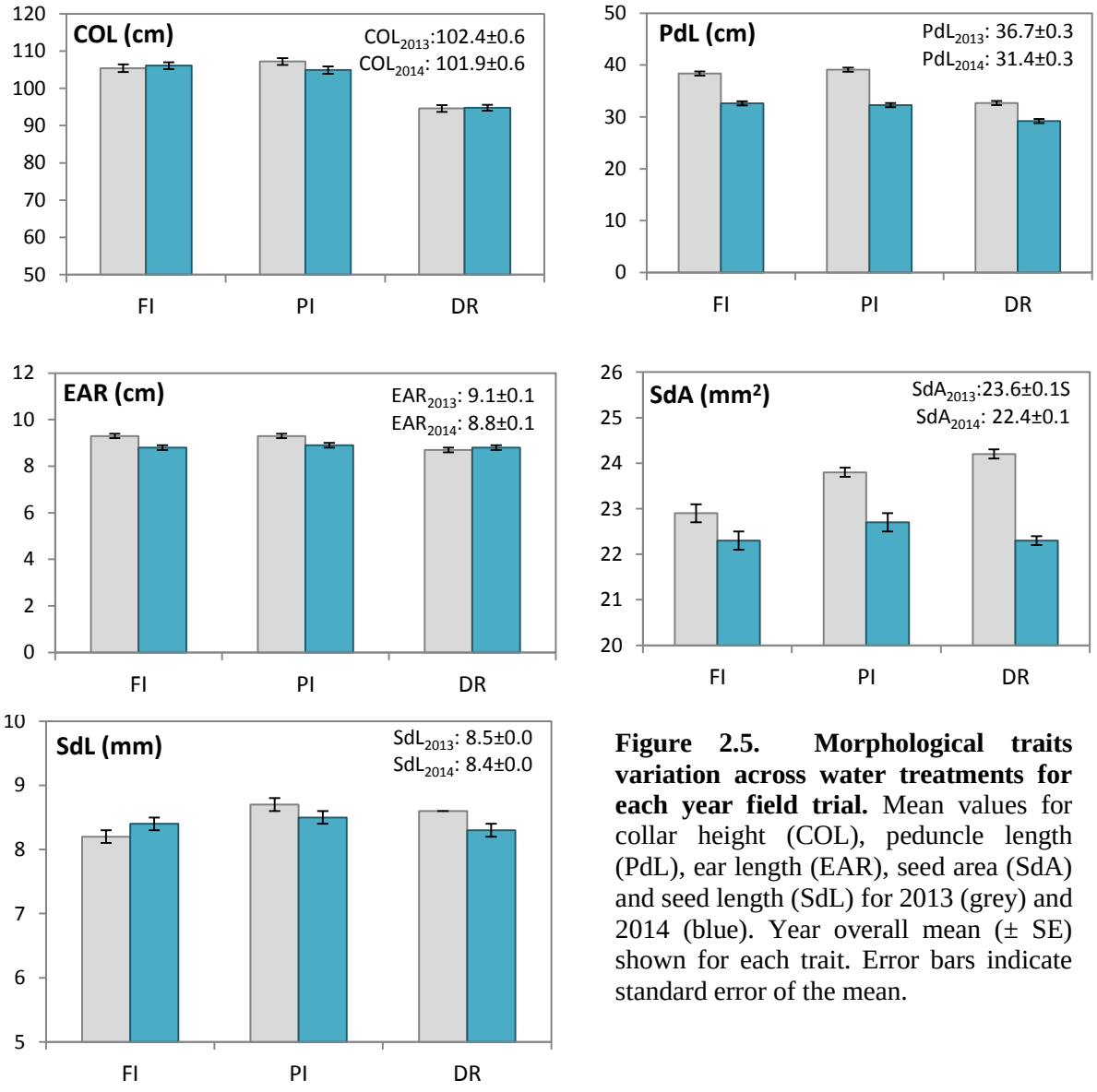

Figure 2.5. Morphological traits variation across water treatments for each year field trial. Mean values for collar height (COL), peduncle length (PdL), ear length (EAR), seed area (SdA) and seed length (SdL) for 2013 (grey) and 2014 (blue). Year overall mean (± SE) shown for each trait. Error bars indicate standard error of the mean.

Peduncle characteristics

The length of the peduncle (PdL and PdE) was also significantly reduced due to the lack of water. In contrast to height, there was a significant effect of year. In 2013, peduncles were longer (36.7 cm) than in 2014 (31.4 cm) (Fig. 2.5). For this trait there was also a wide range of variation within the RCSLs, from 28.7 cm to 64.0 cm under irrigation in 2013, compared to cv. Harrington with values ranging from 28.3 cm to 33.7 cm. The same trend but narrower variation was observed in 2014 (Table 2.4).

Spike characteristics

The RCSLs spike length (EAR) was slightly longer than those of cv. Harrington in all three water treatments across both years (Table 2.4). Overall, spikes were longer in 2013 than in 2014 with mean values of 9.1 cm and 8.8 cm respectively. The effect of the treatment was greater in 2013, mean spike length was 8.7 cm under drought and 9.3 cm in the irrigated plots. The maximum spike length of 13 cm in the partially irrigated plots was observed in 2013 and 2014 (Fig. 2.5).

Seed characteristics

The differences in the seed morphology measurements (SdA, SdL, SdW) are essentially determined by the genotype. The mean value for the RCSLs and cv. Harrington seed area (SdA) was similar in all the water treatments although the maximum values observed in the RCSLs were considerably higher than those in cv. Harrington in all the water treatments (Table 2.4). For example, in 2013 the maximum SdA value in the irrigated plots and drought treatment for the RCSLs were 30.1 mm^2 and 29.1 mm^2 respectively. In contrast, the maximum SdA value in the irrigated plots and drought treatment for cv. Harrington were lower and similar with values of 22.7 mm^2 and 23.0 mm^2 respectively. Similar values were measured in 2014. The water treatment did not have a strong effect on seed parameters. On average, the differences between RCSLs and cv. Harrington for seed length (SdL) and seed width (SdW) were not significant.

ii. Developmental traits

Tiller number

The number of tillers (TILL) was counted on plants harvested from the two middle rows of each plot. Since the sowing density was the same in both field trials and the germination efficiency was high (close to 100%) and did not differ between genotypes, it was assumed that on average the number of plants per plot was the same in both experiments.

Overall, plants in 2013 had significantly more tillers than in 2014, 153.9 compared to 143.0 (Fig. 2.6). Water treatment had a significant effect on the number of tillers. It was observed that the number of tillers decreased with reduced water supply in both field trials. This effect was stronger in 2013 than in 2014. In 2013 the average for number of tillers in partially irrigated conditions and drought conditions was respectively 177.7 and 114.4. In 2014 these mean values were respectively 155.9 and 128.8. On average, the mean number of tillers in cv. Harrington plots was generally higher than in the RCSLs plots for all the water treatments and both years (Table 2.5). It is interesting to note that the genotype effect on the number of tillers was highly significant overall but the interaction between the genotype and the treatment was not significant.

Days to heading

A major difference between 2013 and 2014 was the days to heading (Fig. 2.6). In 2014 the average days to heading was of 59.2 DAS whereas in 2013 this was 64.0 DAS. Water treatments had only a minor effect on this trait. Significant effect of water treatment on days to heading were only observed in 2013, where the fully irrigated plots had a mean value of 64.9 DAS compared to the other two water treatments with values of 63.8 DAS (Part. Irr) and 63.4 DAS (Dr). The genotypic effect accounted for the remaining variation in days to heading. On average, the days to heading for the RCSLs and cv. Harrington were very similar; however, there was greater variation in days to heading within the RCSLs group (Table 2.5). For example, the difference between the earliest and latest days to heading for RCSLs in fully irrigated plots was 18 days in 2013 and 11 days in 2014.

Table 2.5. Developmental traits. Means (± SE) and maximum and minimum range values (in brackets) for tiller number (TILL) and Heading date (HEA) for cv. Harrington (Harr) and the RCSLs under full irrigation (FI), partial irrigation (PI) and drought (DR) conditions in two years field trials (2013 and 2014).

Trait	2013		2014		Effect [1]
Treatment	Harr	RCSL	Harr	RCSL	
TILL					
FI	169.0±10.2	169.7±2.5	140.3±10.8	144.5±2.1	Y ***
	(153.0–197.0)	(105.0–245.0)	(121.0–169.0)	(70.0–202.0)	T ***
PI	182.5±10.1	177.6±2.3	160.8±8.9	155.7±2.1	G ***
	(167.0–212.0)	(110.0–243.0)	(145.0–183.0)	(105.0–207.0)	Y × T ***
DR	120.8±2.8	114.2±1.6	132.8±13.7	128.6±1.9	Y × G *
	(116.0–129.0)	(69.0–157.0)	(112.0–171.0)	(75.0–191.0)	T × G ns
					Y × T × G ns
HEA (DAS)					
FI	65.8±0.3	64.9±0.3	60.5±0.3	59.1±0.2	Y ***
	(65.0–66.0)	(56.0–73.0)	(60.0–61.0)	(52.0–62.0)	T **
PI	65.0±0.4	63.7±0.2	60.7±0.3	59.2±0.2	G ***
	(64.0–66.0)	(55.0–69.0)	(60.0–61.0)	(52.0–63.0)	Y × T *
DR	64.0±0.4	63.4±0.2	60.3±0.5	59.1±0.2	Y × G ***
	(63.0–65.0)	(56.0–68.0)	(59.0–61.0)	(52.0–63.0)	T × G **
					Y × T × G ns

[1] Statistically significant effect of the year (Y), water treatment (T), the genotype (G) and their interaction (YxT, YxG, TxG, YxTxG) on each trait performance. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

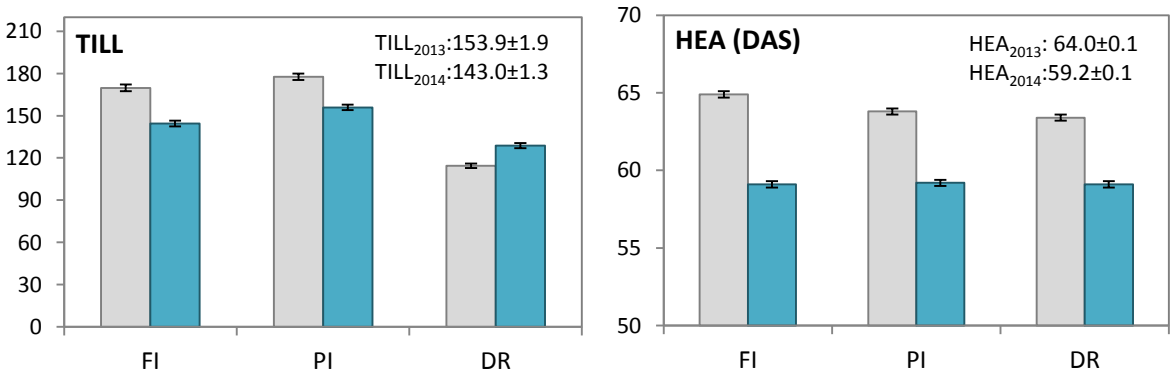

Figure 2.6. Developmental traits variation across water treatments for each year field trial. Mean values for tiller number (TILL) and Heating date (HEA) for 2013 (grey) and 2014 (blue). Year overall mean (± SE) shown for each trait. Error bars indicate standard error of the mean.

iii. Agronomic traits

The effect of year was highly significant (P<0. 001) for all of the agronomic traits measured (Table 2.6). Both cv. Harrington and RCSLs genotypes grew better in 2013 than in 2014. Overall mean values for Dry yield, TGW, Biomass yield and Harvest index were significantly higher in 2013 than those in 2014 (Fig. 2.7). For instance, mean dry yield in 2013 was 3485.5 kg ha^{-1}, significantly larger than the 2014 value of 2825.5 kg ha^{-1}. Additionally, the statistical model including both years revealed significant differences (P<0.001) for year × treatment interaction for both Dry yield and TGW. For this reason, in order to define the effect of the treatment overall, the years were also considered separately using simpler statistical models (Appendix 6).

In 2013, the overall mean dry yield under partial irrigation was 3923.5 kg ha^{-1}, significantly greater than the mean dry yield in the fully irrigated plots (3673.0 kg ha^{-1}). The same effect on yield was found in the irrigated treatments in 2014 although the average yield values obtained in 2014 were significantly lower than in 2013. The yield of partially irrigated plots in 2014 was on average 3101.5 kg ha^{-1} being significantly larger than the mean yield in fully irrigated plots (2735.1 kg ha^{-1}). As expected, plants grown under drought conditions yielded less than plants grown under irrigated conditions in both 2013 and 2014. The mean values for crop yield in drought conditions were 2860.1 kg ha^{-1} in 2013 and 2642.4 kg ha^{-1} in 2014. However, the difference in mean dry yield between the drought and the fully irrigated treatment in 2014 was less than in 2013. Because of this, the analysis of 2013 dry yield values revealed a significant effect not only at the genotypic and treatment level (P<0.001), but also at the genotype × treatment interaction level (P<0.01), suggesting that the RCSLs responded differently to the lack of water in 2013. However, the analysis of the 2014 dry yield values showed that the differences were essentially due to the genotype and the treatment (P<0.001) since the genotype × treatment interaction did not have a significant effect for this trait (p=0.072) (Appendix 6). Nevertheless, the analysis over the two growing seasons defines considerable differences in agronomic performance of the RCSLs depending on the water regime (P<0.001) (Table 2.6). The variation in the RCSLs response will be presented in section 2.3.2.4.

Table 2.6. Agronomic traits. Means (± SE) and maximum and minimum range values (in brackets) for dry yield (DY), thousand grain weight (TGW), Harvest index (HI) and biomass yield (BY) for cv Harrington (Harr) and the RCSLs under full irrigation (FI), partial irrigation (PI) and drought (DR) conditions in two years field trials (2013 and 2014).

Trait / Treatment	2013		2014		Effect[1]
	Harr	RCSL	Harr	RCSL	
DY (kg ha^{-1})					
FI	4207.3±204.7	3653.9±61.1	2723.4±238.6	2735.6±50.2	Y ***
	(3703.5–4705.5)	(2266.8–6064.8)	(2213.0–3354.3)	(1288.6–4234.3)	T ***
PI	4316.2±97.3	3909.5±56.7	3397.9±167.6	3090.8±53.2	G ***
	(4134.5–4526.0)	(2574.5–5727.0)	(3071.5–3794.6)	(1997.3–5072.3)	Y × T ***
DR	3250.9±164.1	2846.1±38.5	3010.7±302.2	2629.2±46.3	Y × G ns
	(3004.8–3725.0)	(1930.8–3845.3)	(2568.6–3894.8)	(1151.8–4294.2)	T × G ***
					Y × T × G *
TGW (g)					
FI	45.8±0.4	45.9±0.3	38.9±0.9	43.0±0.3	Y ***
	(45.0–46.9)	(37.7–54.0)	(36.9–40.9)	(35.0–48.8)	T ***
PI	45.0±0.9	47.2±0.3	39.9±0.9	44.0±0.3	G ***
	(43.2–47.1)	(41.3–54.1)	(37.7–42.0)	(36.0–52.9)	Y × T ***
DR	46.3±0.9	48.1±0.2	46.6±1.4	47.6±0.3	Y × G ***
	(44.2–48.4)	(42.2–55.9)	(43.1–49.6)	(39.8–56.1)	T × G ***
					Y × T × G ***
HI (%)					
FI	47.5±0.6	41.3±0.4	44.5±0.4	40.2±0.5	Y ***
	(46.2–48.9)	(27.9–48.8)	(43.6–45.0)	(28.8–50.2)	T ***
PI	47.4±0.9	43.0±0.3	43.6±1.8	40.9±0.4	G ***
	(45.9–49.9)	(33.1–50.3)	(39.7–47.5)	(32.0–48.3)	Y × T ns
DR	47.9±0.6	43.4±0.3	45.1±2.0	42.4±0.4	Y × G ***
	(46.6–49.0)	(35.4–51.2)	(39.8–48.8)	(19.0–51.0)	T × G **
					Y × T × G ns
BY (kg ha^{-1})					
FI	8862.5±412.4	8822.1±107.5	6225.0±694.6	6813.0±123.3	Y ***
	(7875.0–9850.0)	(6025.0–13450.0)	(5075.0–7475.0)	(3925.0–10350.0)	T ***
PI	9131.3±332.2	9083.3±107.9	7787.5±210.3	7547.7±106.1	G ***
	(8350.0–9850.0)	(6300.0–11,850.0)	(7400.0–8275.0)	(5150.0–11050.0)	Y × T ***
DR	6793.8±396.9	6567.6±82.9	6700.0±711.3	6210.5±88.9	Y × G ns
	(6150.0–7875.0)	(4350.0–8675.0)	(5650.0–8775.0)	(4250.0–10425.0)	T × G ns
					Y × T × G ns

[1] Statistically significant effect of the year (Y), water treatment (T), the genotype (G) and their interaction (YxT, YxG, TxG, YxTxG) on each trait performance. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

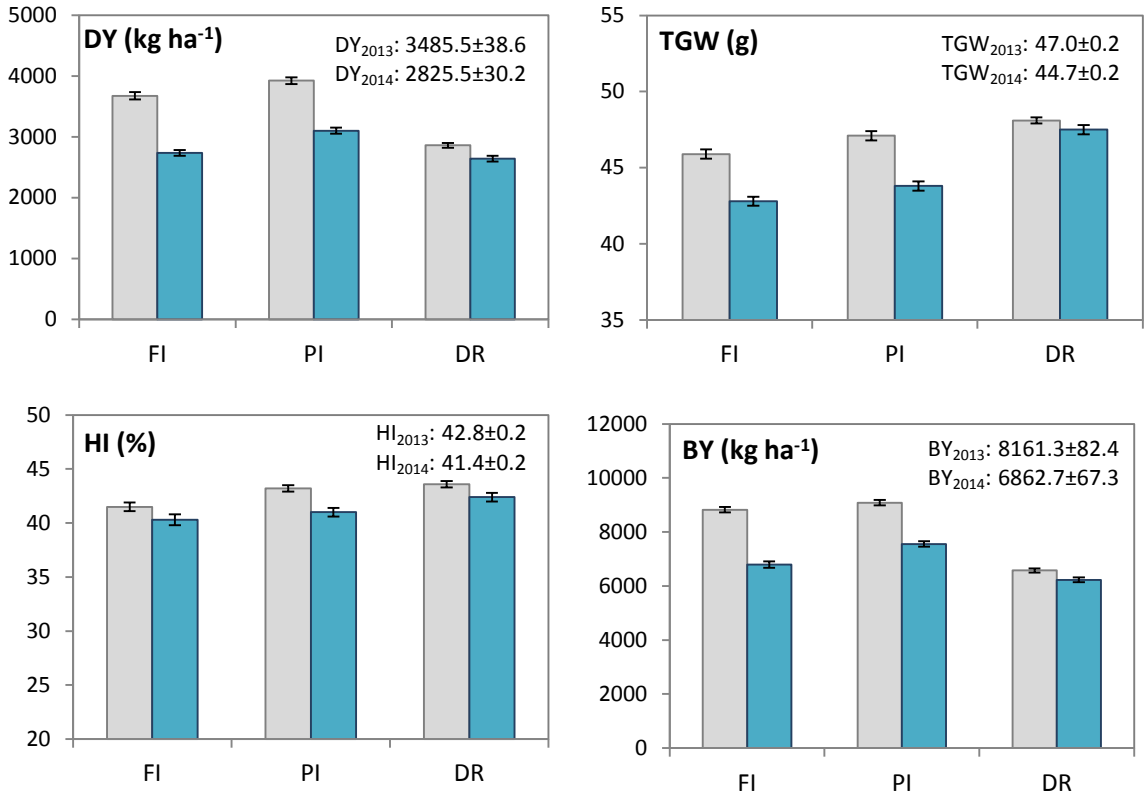

Figure 2.7. Agronomic traits variation across water treatments for each year field trial. Mean values for dry yield (DY), thousand grain weight (TGW), Harvest index (HI) and biomass yield (BY) for 2013 (grey) and 2014 (blue). Year overall mean (± SE) shown for each trait. Error bars indicate standard error of the mean

Interestingly, the mean values for TGW under drought were consistently larger than the TGW values recorded under partial or full irrigated in both years of the field trial. Mean values of TGW in droughted plots was 48.1 g and 47.5 g in 2013 and 2014 respectively, in contrast the mean TGW values obtained in the partially irrigated plots were significantly lower, with mean TGW values of 47.1 in 2013 and 43.8 g in 2014. The mean TGW values obtained in the fully irrigated plots were significantly lower than the TGW recorded under both drought conditions and partial irrigation across both years, with a mean TGW of 45.9 g in 2013 and 42.8 g in 2014. Similarly to the response observed for DY, TGW was significantly larger in 2013 than in 2014, 47.0 and 44.7 mean values respectively.

Under the experimental conditions, full irrigation of the plots appeared to be sub-optimal for crop growth and yield. The partial irrigation treatment however was more appropriate than all other treatments producing higher yields in both field trials and thus, providing more appropriate potential yield values for the experimental conditions. It is likely that full irrigation delivered excessive amounts of water in the soil, causing hypoxia and waterlogging while complete removal of water supply induced drought stress and increased soil strength. For this

reason, partial irrigated plots will be considered as the control treatment and will also be termed the "well-watered" (WW) treatment in this work.

2.3.2.2 Phenotypic correlation among investigated traits

Pearson's correlation coefficients (*r*) between the 14 traits measured were calculated in order to understand the nature of the variation in the traits within the RCSLs population. Calculation of Pearson's correlation coefficients used the trait values for all genotypes across the two years field trials separately, both for the control or well-watered plots (WW) and water stress conditions (WS) (226 and 229 observations respectively). In addition, the autocorrelation coefficients of each trait between the well-watered and water stress condition were determined using the genotype mean values across each water regime and field trial.

A total of 123 significant correlations were detected between the 14 traits analysed, 57 in the water stress treatment and 66 in the control conditions (Table 2.7). Highly related traits showed the strongest positive correlations as the ones found between collar height and total height ($r=1$ in both WW and WS, $P<0.001$), peduncle length and peduncle extrusion ($r=0.96$ in WW and $r=0.95$ in WS, $P<0.001$), biomass yield and dry yield ($r=0.88$ in WW and $r=0.83$ in WS, $P<0.001$) and seed length and seed area ($r=0.90$ in WW and $r=0.85$ in WS, $P<0.001$).

In summary, other strong positive correlations were found between seed area and TGW ($r=0.68$ in WW and $r= 0.62$ in WS, $P<0.001$) and between peduncle length and total height ($r=0.56$ in WW and $r=0.60$, $P<0.001$). Significative negative correlation was found between harvest index and collar height ($r=-0.48$ in WW and $r=-0.46$ in WS, $P<0.001$). For days until heading, positive correlations were determined for plant height ($r=0.32$) and ear length ($r=0.25$) under well-watered conditions ($P<0.001$). However, these associations were not highly significant or were not found under water-stress ($r=0.19$, $P<0.01$ for collar height, ns for ear length). Dry yield revealed highly significant positive correlations ($P<0.001$) with the number of tillers ($r=0.62$), harvest index ($r=0.61$), days to heading ($r=0.51$), TGW ($r=0.42$), seed width ($r=0.33$) and peduncle length ($r=0.30$) in the well-watered condition. These correlations were highly significant ($P<0.001$) under water stress between dry yield and number of tillers ($r=0.51$), harvest index ($r=0.52$) and seed width ($r=0.29$). Correlation between dry yield and days to heading was smaller ($r=0.21$, $P<0.01$) than under control conditions.

Under both water treatments, TGW shows highly positive correlations ($P<0.001$) with seed morphological traits like seed width ($r=0.62$ in WW and $r=0.43$ in WS) and seed area ($r=0.68$ in WW and $r=0.62$ in WS). It is also significantly correlated ($P<0.001$) with the peduncle length in

both water regimes (r=0.48 in WW and r=0.29 in WS) and with ear length under the well-watered treatment (r=0.27). Correlation with days to heading is only significant (P<0.01) under well-watered conditions (r=0.21).

Autocorrelations of traits between control and water stress treatment were highly significant (P<0.001) for all the traits apart from number of tillers, which was not significant. The highest correlations were found for days to heading (r=0.98) and height (r=0.90).

Table 2.7. Pearson's correlation coefficient (*r*) between 14 traits measured in the 2013 and 2014 field trials under WW (well-watered, on the left) and WS (water stress, on the right) conditions.

HEI	***0.90*****	1.00***	0.61***	0.54***	0.50***	-0.11ns	0.19**	0.04ns	0.06ns	-0.46***	0.35***	0.11ns	0.04ns	0.16*
COL	1.00***	***0.90*****	0.60***	0.54***	0.43***	-0.10ns	0.19**	0.05ns	0.05ns	-0.46***	0.36***	0.11ns	0.05ns	0.16*
PdL	0.57***	0.56***	***0.84*****	0.95***	0.32***	-0.10ns	0.17**	0.16*	0.29***	-0.15*	0.29***	0.47***	0.34***	0.32***
PdE	0.43***	0.43***	0.96***	***0.83*****	0.23***	-0.02ns	0.09ns	0.17*	0.24***	-0.11ns	0.26***	0.37***	0.27***	0.28***
EAR	0.58***	0.51***	0.44***	0.31***	***0.70*****	-0.23***	0.08ns	-0.04ns	0.10ns	-0.24***	0.11ns	0.05ns	-0.03ns	0.16*
TILL	-0.08ns	-0.07ns	0.26***	0.32***	-0.18**	***0.22 ns***	-0.39***	0.51***	-0.16*	0.16*	0.49***	-0.31***	-0.16*	-0.24***
HEA	0.33***	0.32***	0.27***	0.21**	0.25***	0.14*	***0.98*****	0.21**	-0.11ns	-0.09ns	0.31***	0.32***	0.07ns	0.41***
DY	0.11ns	0.10ns	0.30***	0.28***	0.13ns	0.62***	0.51***	***0.69*****	0.12ns	0.52***	0.83***	0.07ns	-0.07ns	0.29***
TGW	0.17*	0.15*	0.48***	0.43***	0.27***	0.05ns	0.21**	0.42***	***0.69*****	0.16*	0.03ns	0.62***	0.46***	0.43***
HI	-0.47***	-0.48***	-0.06ns	0.01ns	-0.17**	0.31***	0.08ns	0.61***	0.29***	***0.86*****	-0.05ns	-0.01ns	-0.10ns	0.13ns
BY	0.42***	0.42***	0.42***	0.35***	0.27***	0.59***	0.59***	0.88***	0.36***	0.17**	***0.59*****	0.10ns	-0.01ns	0.25***
SdA	0.08ns	0.07ns	0.36***	0.29***	0.18**	0.00ns	0.04ns	0.13*	0.68***	0.06ns	0.14*	***0.87*****	0.85***	0.46***
SdL	-0.02ns	-0.03ns	0.16*	0.10ns	0.06ns	-0.01ns	-0.09ns	-0.04ns	0.38***	-0.04ns	-0.02ns	0.90***	***0.89*****	0.03ns
SdW	0.25***	0.24***	0.27***	0.22***	0.21**	-0.11ns	0.21**	0.33***	0.62***	0.17**	0.31***	0.30***	-0.05ns	***0.74*****
	HEI	COL	PdL	PdE	EAR	TILL	HEA	DY	TGW	HI	BY	SdA	SdL	SdW

The agronomic traits are defined in Table 2.2. Correlations for each treatment were calculated based on data obtained per plot. The italics values on the diagonal of the table correspond to the autocorrelation coefficients between water stress and well-watered treatments of each single trait. The *r* values are significant with ***P<0.001,**P<0.01 or *P<0.05; ns: not significant

2.3.2.3 Line × phenotype associations

In order to characterize the significantly strong effect of the genotype in the phenotypic variation found for all the traits, it is essential to test whether the wild barley chromosome regions introgressed in cv. Harrington genome are effectively affecting the performance of the RCSLs and in which direction (enhancing or diminishing the trait).

A multi-comparison test between the Best Linear Unbiased Estimates (BLUEs) for genotype and genotype × treatment interaction were achieved with the VMCOMPARISON procedure using the Fisher's LSD test. By considering the cv. Harrington BLUEs as control, RCSLs with a significantly overall improved performance as well as those with a reduced or unaffected phenotype for the experimental conditions, were identified. Presumably, the allelic variation introduced by the donor parent in the RCSLs is responsible for the phenotypic differences observed for the different traits.

i. Morphological traits

For the morphological traits analysed, the majority of the RCSLs had significantly higher mean values when compared to the cultivated parent (HEI, COL, PdL, PdE, EAR, SdA and SdL). Harrington means were smaller than the overall mean of each trait except in case of the seed width (SdW) (Fig. 2.8). Mean SdW for cv. Harrington was 3.55 mm (SdW overall was 3.5 mm). Ten RCSLs had SdW means in the range of cv. Harrington and four out of this group also had mean spike length (EAR) in the range of the recurrent parent (OSU035, OSU074, OSU060, OSU090). The other six RCSLs had significantly longer spikes compared to the elite parent ones (OSU019, OSU048, OSU102, OSU033, OSU061 and OSU024). Interestingly, within the group of significantly long ears we found RCSLs with significantly increased mean seed parameters (SdA, SdW, SdL). For example, OSU040 had EAR mean value of 9.49 cm and mean seed measurements of 24.4 mm for SdA, 3.7 mm for SdW and 8.6 mm for SdL. The mean EAR for cv. Harrington (8.4 cm) was around one centimetre shorter than OSU040 with significantly smaller SdA, SdW and SdL means (21.4 mm, 3.6 mm and 7.9 mm respectively).

The variation in height within the RCSLs was highly significant and consistent across the years, showing the high heritability of this trait. Most of the RCSLs (25 lines) were significantly taller than the cultivated parent (which overall mean COL was 91.7 cm). Nevertheless, RCSLs such as OSU019 and OSU127 were not significantly different from cv. Harrington for HEI and COL and OSU051 was the only line significantly shorter than the recurrent parent (COL 88.8 cm). Despite the fact that these lines seem to have an improved phenotype for height, OSU127 and OSU051 were the only two RCSLs that had significantly shorter spikes when compared to the

elite barley, as well as differences in seed measurements. The range in combinations of variable traits is observed in OSU015 which had the longest (11.08 mm) and narrowest seeds (3.4 mm), resulting in the larger RCSLs seed area measured (SdA=27.8 mm^2).

The tallest RCSLs (OSU024, OSU035, OSU061, OSU040, OSU065 and OSU033) showed significantly longer peduncles (PdL) than the recurrent parent (28.7 cm). Interestingly, OSU065 had also the longest spikes (10.4 cm) and seed measurements (SdA=24.6 mm^2). Spike length and seed sizes were considerably high compared to the other RCSLs and Harrington. However, this line was very sensitive to lodging. Other lines like OSU040 had similar mean height values and seed measurements to OSU065 without showing severe lodging problems.

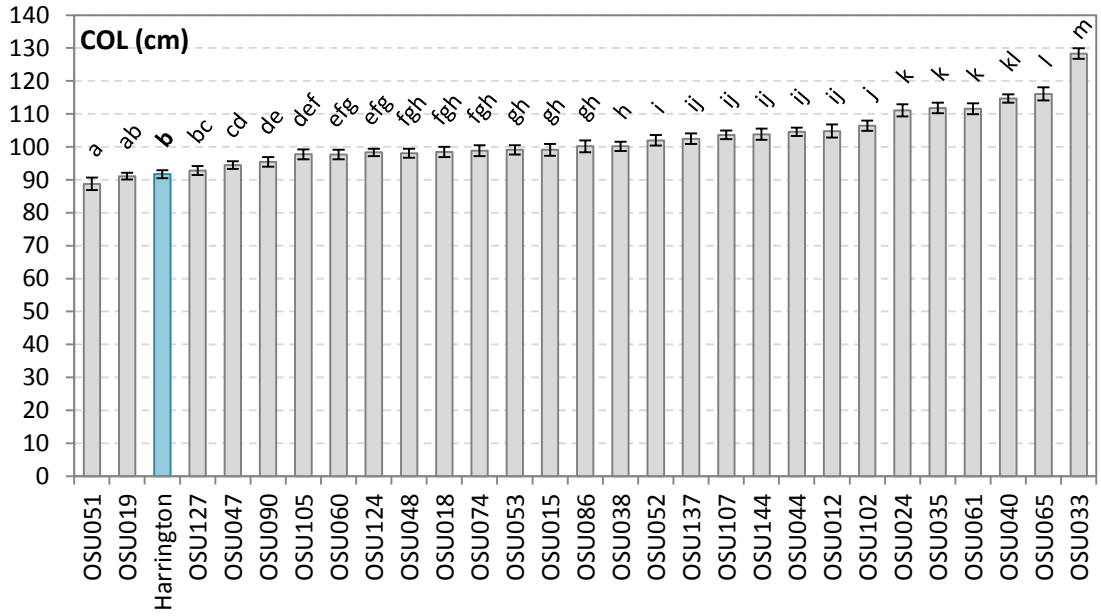

Figure 2.8. Morphological traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means for collar height (COL), peduncle length (PdL), ear length (EAR), seed area (SdA) and seed width (SdW). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly (P<0.01, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

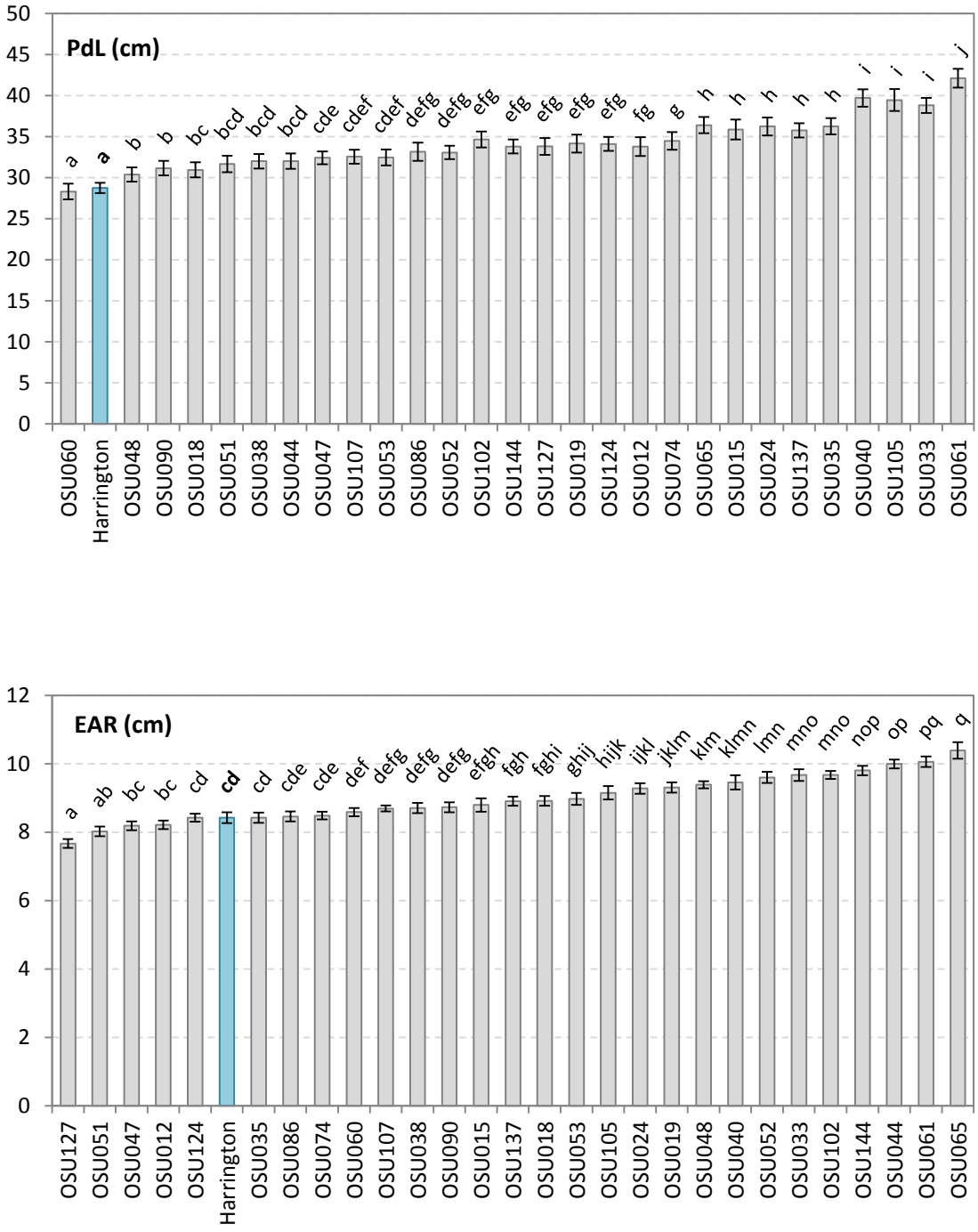

Figure 2.8 (continued). Morphological traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means for collar height (COL), peduncle length (PdL), ear length (EAR), seed area (SdA) and seed width (SdW). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly ($P<0.01$, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

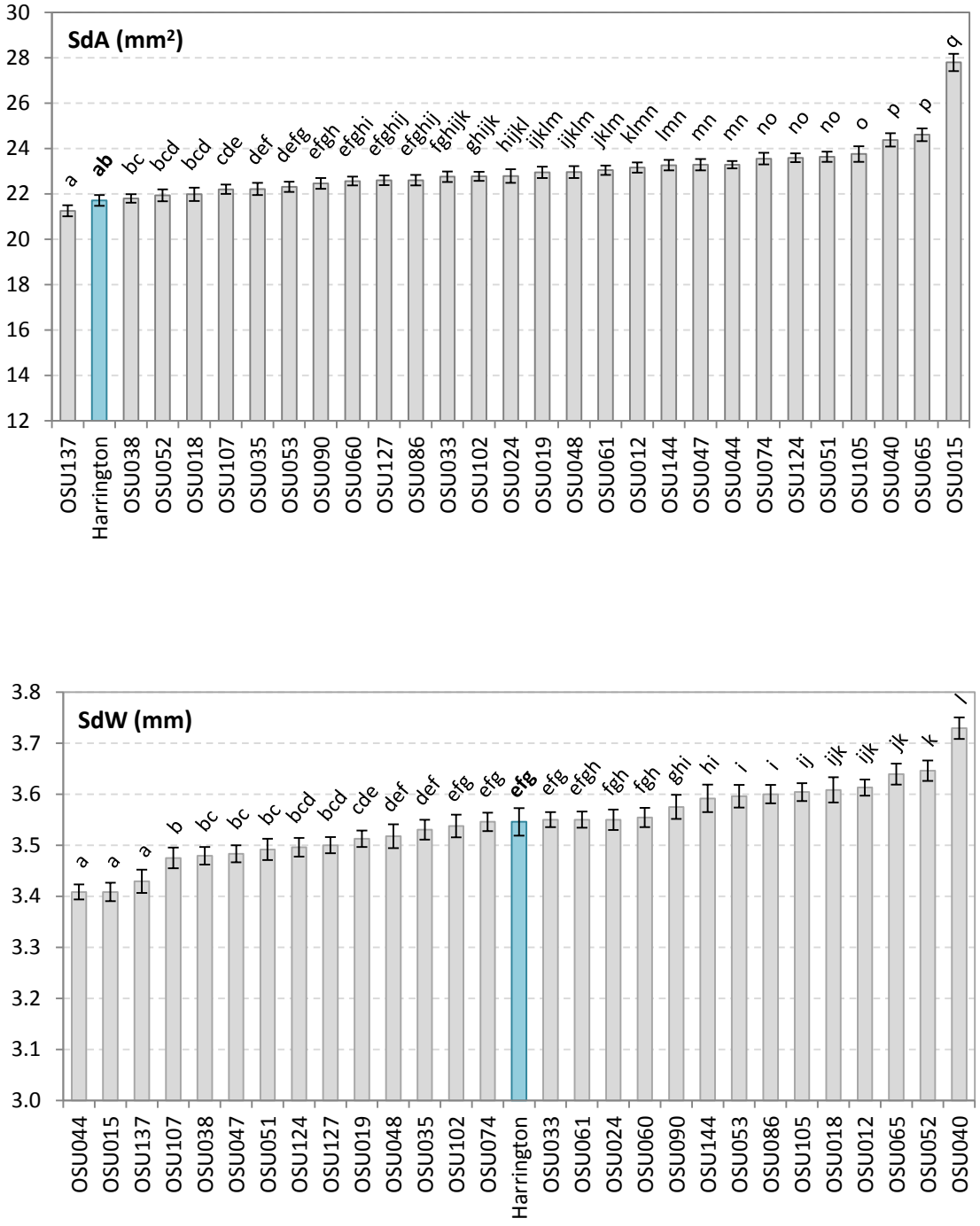

Figure 2.8 (continued). Morphological traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means for collar height (COL), peduncle length (PdL), ear length (EAR), seed area (SdA) and seed width (SdW). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly ($P<0.01$, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

ii. Developmental traits

For number of tillers (TILL) and days to heading (HEA) (Fig. 2.9), cv. Harrington mean values were larger than the overall mean of the trait. 14 RCSLs had significantly fewer number of days to heading, earlier flowering, than cv. Harrington (62.7 DAS). The genotypes with the shortest days to heading were OSU105 (54.6 DAS), OSU019 (57.3 DAS), OSU127 (57.7 DAS), OSU015 (60.0 DAS) and OSU047 (60.2 DAS). In contrast, a group of four RCSLs had significantly more number of days to heading compared to the elite parent: OSU107 (63.3 DAS), OSU052 (63.6 DAS), OSU048 (63.3 DAS) and OSU065 (64.8 DAS). Interestingly, these last three lines had significantly lower mean tiller number than the rest of the genotypes with values of 139.4, 136.5 and 115.0 (mean TILL for cv. Harrington was 151.0). Two of the lines which flowered earlier, OSU47 (60.2 DAS) and OSU127 (57.7 DAS), were the only two RCSLs with significantly more tillers than cv. Harrington, with 170.3 and 176.0 tillers, respectively.

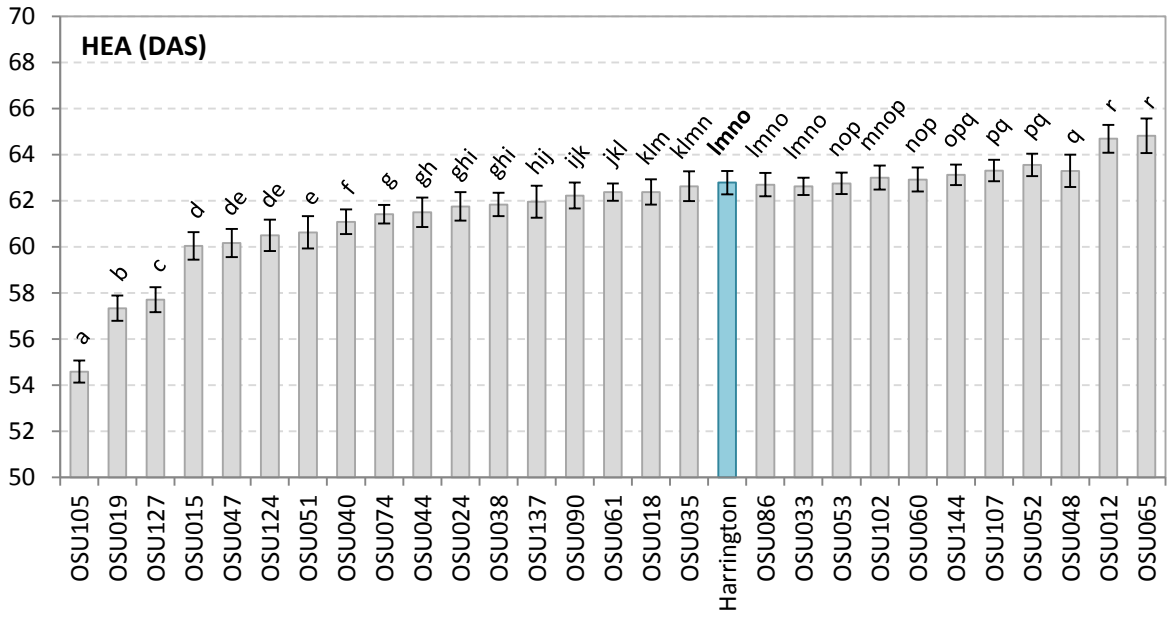

Figure 2.9. Developmental traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means for heading date (HEA) and tiller number (TILL). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly (P<0.01, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

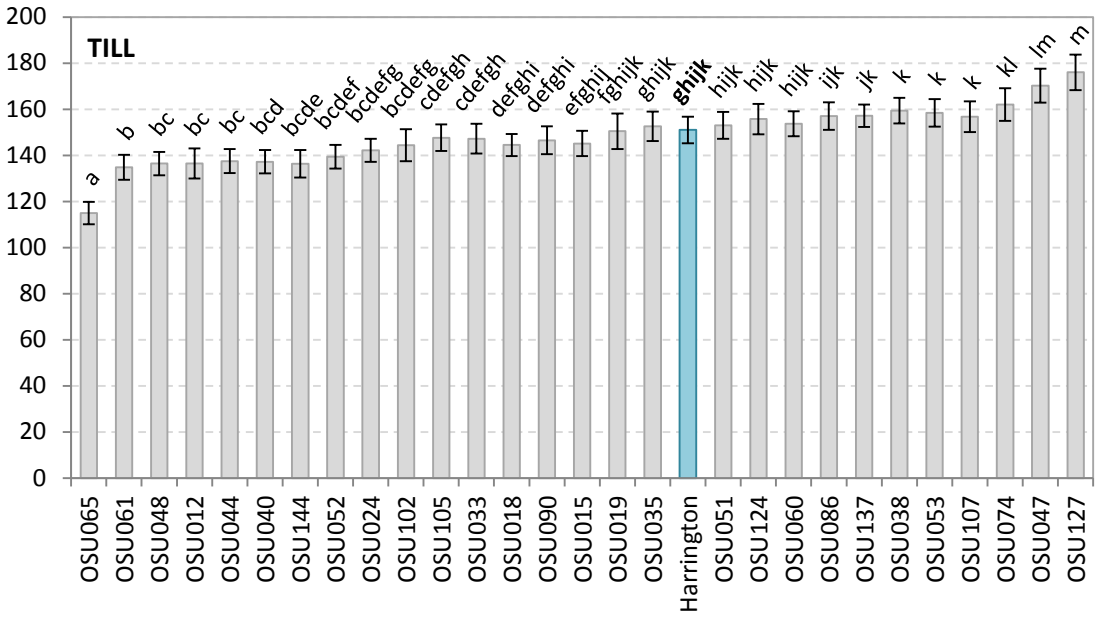

Figure 2.9 (continued). Developmental traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means for heading date (HEA) and tiller number (TILL). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly (P<0.01, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

iii. Agronomic traits

For dry yield (DY), biomass yield (BY) and harvest index (HI), the means for cv. Harrington (3484.4 kg ha^{-1}, 7642.4 kg ha^{-1} and 45.6%) were larger than the overall mean accounted for each trait for all lines (3155.9 kg ha^{-1}, 7542.3 kg ha^{-1} and 41.8%). None of the RCSLs had significantly larger DY or HI. OSU053 was the only RCSL with a significantly larger BY than the elite parent with a mean value of 8239.1 kg ha^{-1}. However, the mean TGW of cv. Harrington (43.6 g) was smaller than the overall mean value of the trait for the entire population (45.8 g) being significantly smaller than 22 RCSLs values for TGW (Fig. 2.10). OSU015, OSU105 and OSU040 were the RCSLs with the largest values for TGW: 48.8 g, 49.6 g and 50.4 g.

Ten RCSLs had DY values comparable to cv. Harrington. Their performance overall was not significantly reduced and some of these lines, like OSU060 (3619.7 kg ha^{-1}) and OSU053 (3715.5 kg ha^{-1}) had mean DY values greater than cv. Harrington (3484.4 kg ha^{-1}) although not significantly different. These two RCSLs also had values of HI in the range of cv. Harrington; however, they had a significantly improved TGW value compared to the elite barley (45.3 g for OSU053 and 47.2 g for OSU060). Similarly OSU040 had DY values in the range of cv. Harrington (3408.6 kg ha^{-1}) but had the highest mean value for TGW, 50.4 g. Together with

OSU040, OSU015 and OSU105 had significantly the largest TGW mean values, 48.9 g and 49.6 g respectively, however, in this case the two lines were poor in terms of yield with much lower values than the elite barley (2763.1 kg ha^{-1} for OSU015 and 2842.4 kg ha^{-1} for OSU105).

Thirteen out of 18 significantly low yielding RCSLs had high biomass yield values, comparable to cv. Harrington, however, they had the lowest harvest index values. For example, OSU033 had the lowest overall HI (34.3%) despite the fact that its BY was 8278.0 kg ha^{-1}. Harvest index measures the ratio of harvested grain to total aboveground biomass. Therefore, in this case the proportion of photosynthate allocated to the vegetative parts of the plant was large compared to the one for grain, possibly due to the fact that these genotypes were significantly taller or developed more infertile tillers.

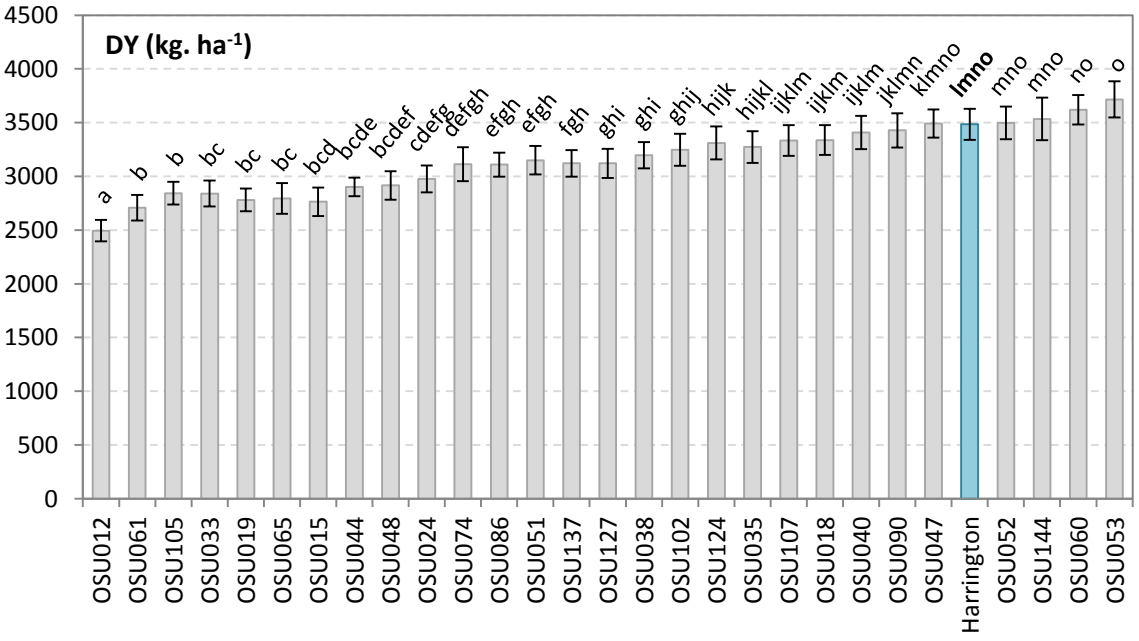

Figure 2.10. Agronomic traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means dry yield (DY), thousand grain weight (TGW), Harvest index (HI) and biomass yield (BY). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly ($P<0.01$, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

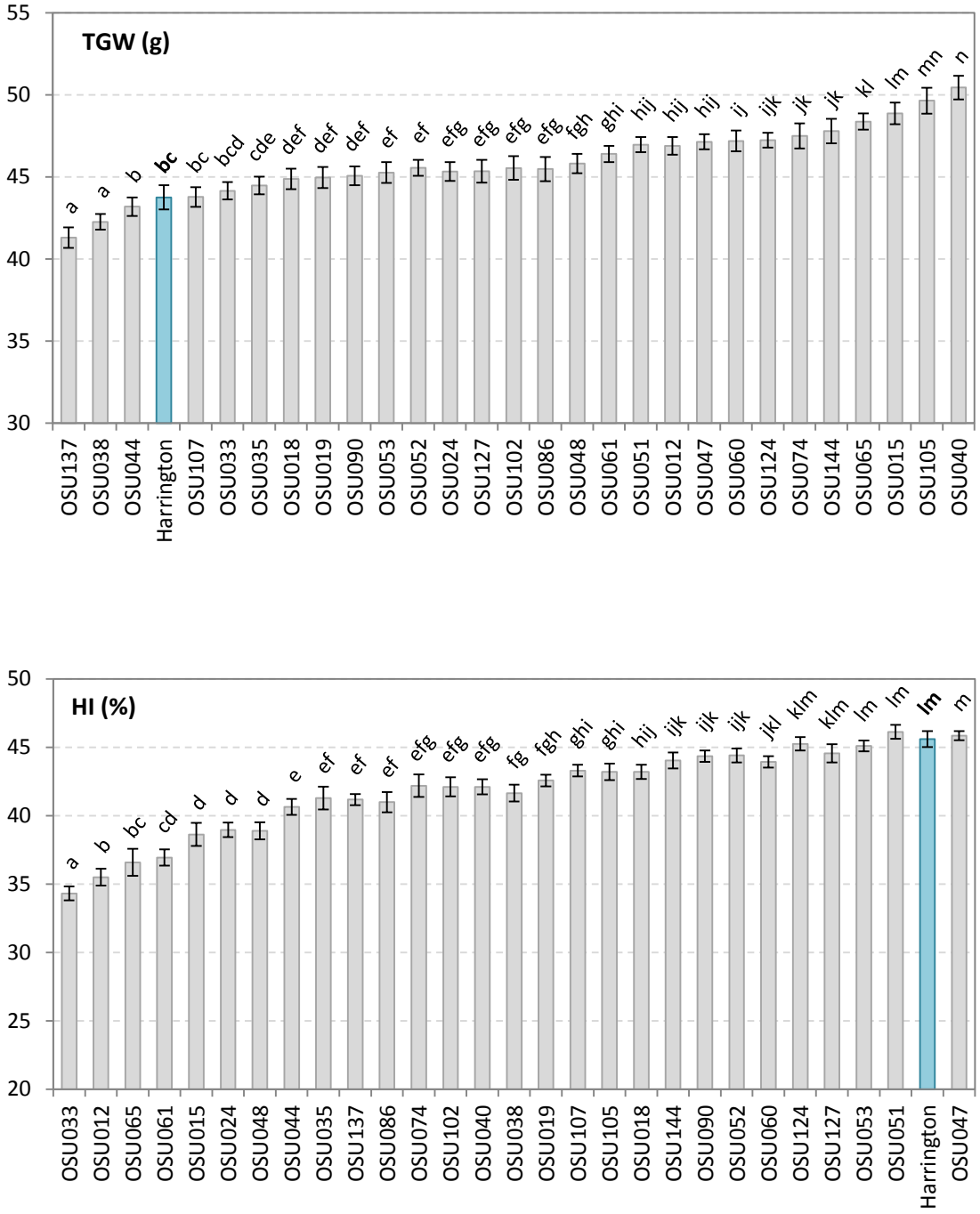

Figure 2.10 (continued). Agronomic traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means dry yield (DY), thousand grain weight (TGW), Harvest index (HI) and biomass yield (BY). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly ($P<0.01$, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

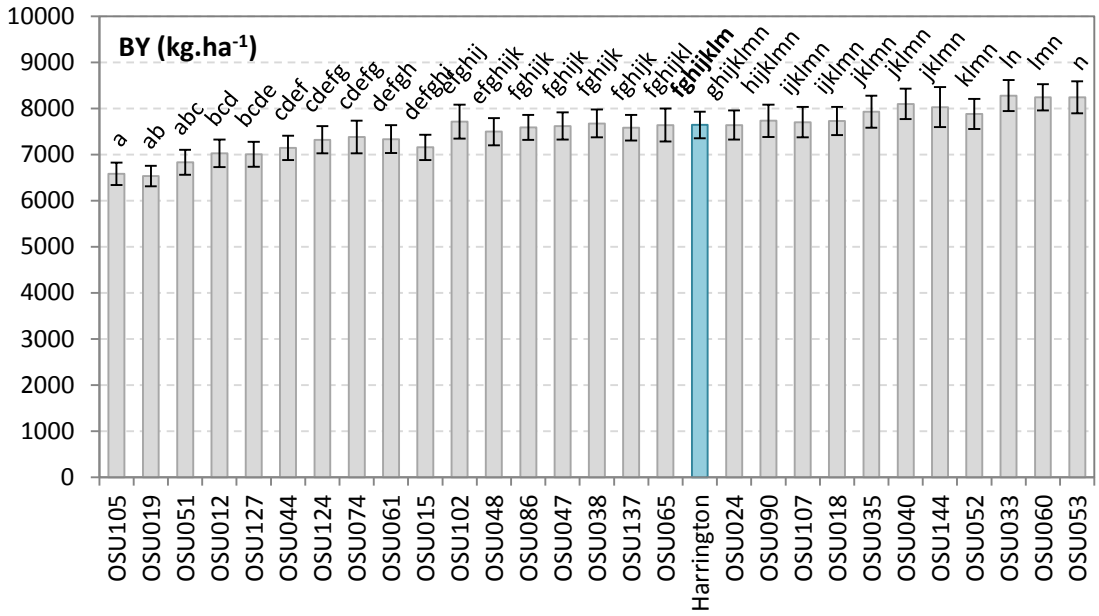

Figure 2.10 (continued). Agronomic traits genotypic variation for RCSLs (grey bars) and cv. Harrington (blue bar). Genotypes are arranged in ascending order of genotypic means dry yield (DY), thousand grain weight (TGW), Harvest index (HI) and biomass yield (BY). Best linear unbiased estimators (BLUEs) for genotypes labelled with different letters differ significantly ($P<0.01$, Fisher's LSD multi-comparison test). Error bars correspond to standard error of the mean.

iv. RCSLs contrasted phenotypes

The analysis of the overall genotype performance across years and water treatments shows a wide range of genotypic variation. To summarise, the following section groups RCSLs and compares and contrasts morphological, developmental and agronomic traits with the cultivar Harrington.

Genotypes such as OSU019 and OSU060 were not significantly different from cv. Harrington in their morphology (HEI, COL, PdL, SdW) and development (TILL), however, such lines differed remarkably in their agronomic performance overall. Despite the fact that OSU019 had a slightly enhanced TGW, this genotype had much reduced yield, a significantly low biomass yield and therefore a low harvest index. In contrast, OSU060 did not show a reduced agronomic potential compared to cv. Harrington, but showed a significantly improved TGW mean value. The significant difference in days to heading (HEA) of the two lines (OSU019 is one of the

earliest RCSLs whereas OSU060 is similar cv. Harrington) might be related to the overall agronomic performance due to the experimental conditions.

OSU052 and OSU065 were two tall genotypes (HEI, COL, PdL) exhibiting a significantly greater number of days to heading and with longer spikes (EAR). Despite these morphological and developmental similarities, OSU052 was a high yielding genotype with agronomic traits such as DY, BY and HI reaching values similar to those observed in the elite parent, cv. Harrington. By contrast, OSU065 did not maintain any of the agronomic characteristics of the elite and exhibited much reduced yield. Both genotypes had improved TGW overall.

Other lines showed developmental traits that were not significantly different from cv. Harrington but had morphological traits values much larger than the elite parent. OSU033 and OSU035, for instance, had a similar mean TILL and HEA to the elite parent but had larger HEI, COL and PdL. Despite the similarities between them, OSU035 reached mean values of DY, TGW and BY comparable to cv. Harrington, whereas OSU033 showed a significantly reduced overall performance and was one of the lowest yielding RCSLs.

The wide genotypic variation found for key morphological, developmental and agronomic traits in the set of 28 RCSLs makes this small group of lines an interesting material to define the main chromosome regions involved in the phenotypic variation observed. Additionally, the RCSLs were found to diverge from the elite parent in qualitative measurable traits such as spike glossiness, lodging, seed shattering, grain threshability and others characters such as auricule colour, leaf rolling and spike purple coloration (Table 2.8, Figure 2.11)

Table 2.8. Phenotypic variation for visually scored qualitative traits

Trait	Genotypes
Spike glossiness	OSU012, OSU047, OSU060, OSU074 and OSU090
Lodging	OSU065
Seed shattering	OSU012
Grain threshability	OSU015
White auricule	OSU012, OSU019, OSU065, OSU105, OSU127, OSU137 and OSU144
Purple grains	OSU012, OSU065, OSU144

Figure 2.11 Phenotypic diversity for some qualitative traits. (A) OSU105 non-glossy spike, (B) OSU074 glossy spike, (C, D) OSU144 and OSU 012 purple spikes, (E) cv. Harrington pink auricle, (F) OSU 127 white auricle.

2.3.2.4 Measuring impact of drought on yield across the RCSLs

This section focuses on the analysis of the response to water stress across the RCSLs and the differences of its impact on agronomic performance (Genotype × Treatment interaction). Generally, genotypes with high yield potential in well-watered conditions tended to yield well under water stress (Fig. 2.12). Despite this general trend, some genotypes were more responsive to the environmental conditions than others. Genotype × treatment interaction effect was highly significant for agronomic traits as dry yield (DY) and TGW. This response was analysed using the drought tolerance index (DTI) and genotype by environment statistical analysis using the AMMI model approach.

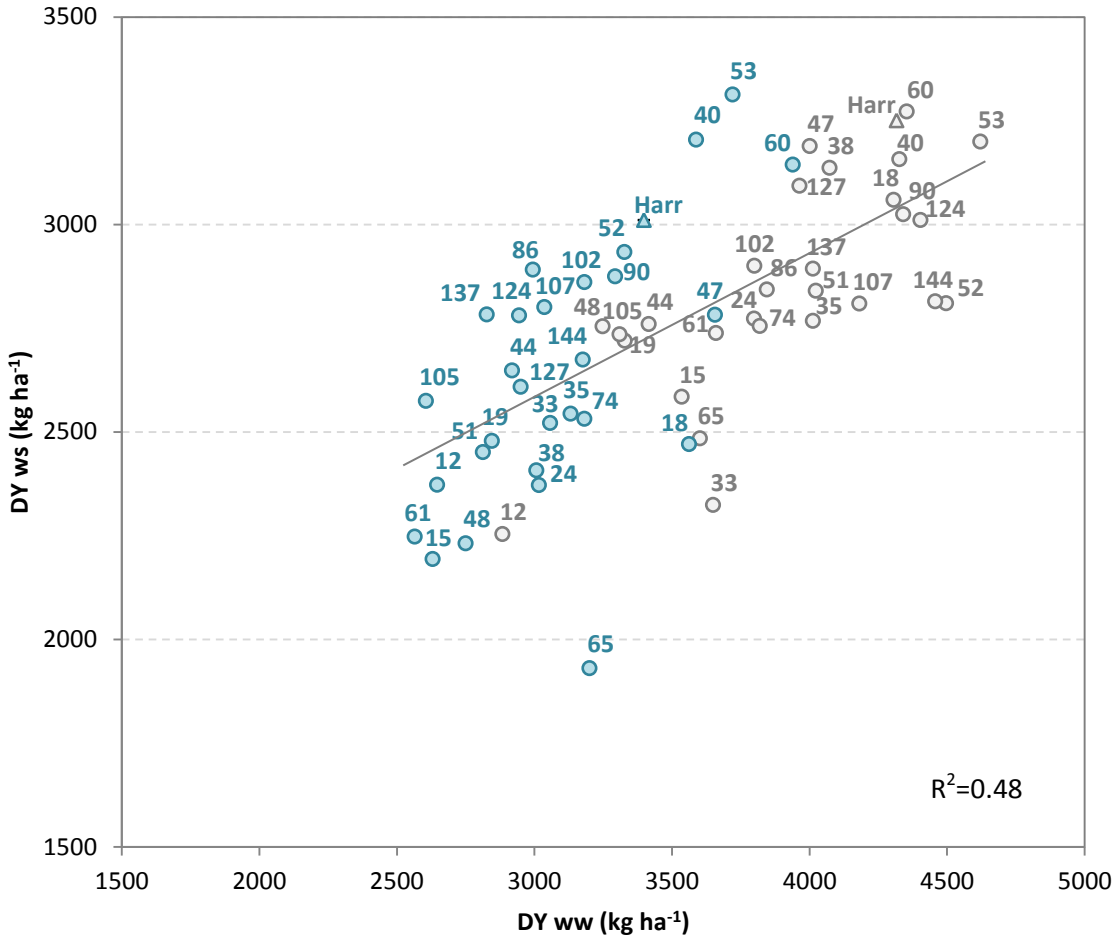

Figure 2.12. RCSLs and cv Harrington mean yields under drought conditions (DYws, y axes) represented against RCSLs yield potential under favourable conditions (DYww, x axes) in 2013 (grey dots) and 2014 (blue dots). Data-points numeration correspond to the RCSLs (OSU) number; cv. Harrington shown as "Harr". Fitted line by linear regression ($P<0.001$)

Even though 2014 was significantly lower yielding compared to 2013 and the effect of water stress was greater in 2013, the genotypes performed similarly across field trials, the genotype × year interaction was therefore not significant for this trait (Table 2.6). However, the variation in the RCSLs response to the water regimes in 2013 was greater than in 2014 due to the more favourable conditions in 2013 for testing the effect of drought on yield. Nevertheless, as described in section 2.3.2.1, full watering in the experimental set-up was detrimental for crop production in both field experiments. For measuring the impact of drought (DTI), partially irrigated plots were therefore considered as the well-watered condition (WW) or control to define the effect of water stress (WS) on performance. On the other hand, for the study of genotype by environment interaction the three water regimes were considered to define six different environments in combination with the growing season.

i. Drought tolerance index (DTI)

Drought tolerance index (DTI) was used to determine the relative stress response of all genotypes and it was calculated across growing seasons referred to as the overall Drought Intensity Index (DII) which measures the general effect of drought on crop production across genotypes and years. Overall dry yield values for the WW and WS condition were used in the calculation of DTI (Fig. 2.13). RCSLs with DTI values equal to 1 or larger, were considered as drought tolerant. The value of DII was 0.78 indicating that the overall decrease in yield due to the lack of water was 22%. DTI ranged from 0.82 to 1.15, with OSU065, the most susceptible, and OSU105 most tolerant line. Harrington was among the group of sixteen tolerant lines with a DTI value of 1.04. Thirteen lines had DTI values smaller than 1 and therefore were considered as more susceptible to the water stress for the experimental conditions.

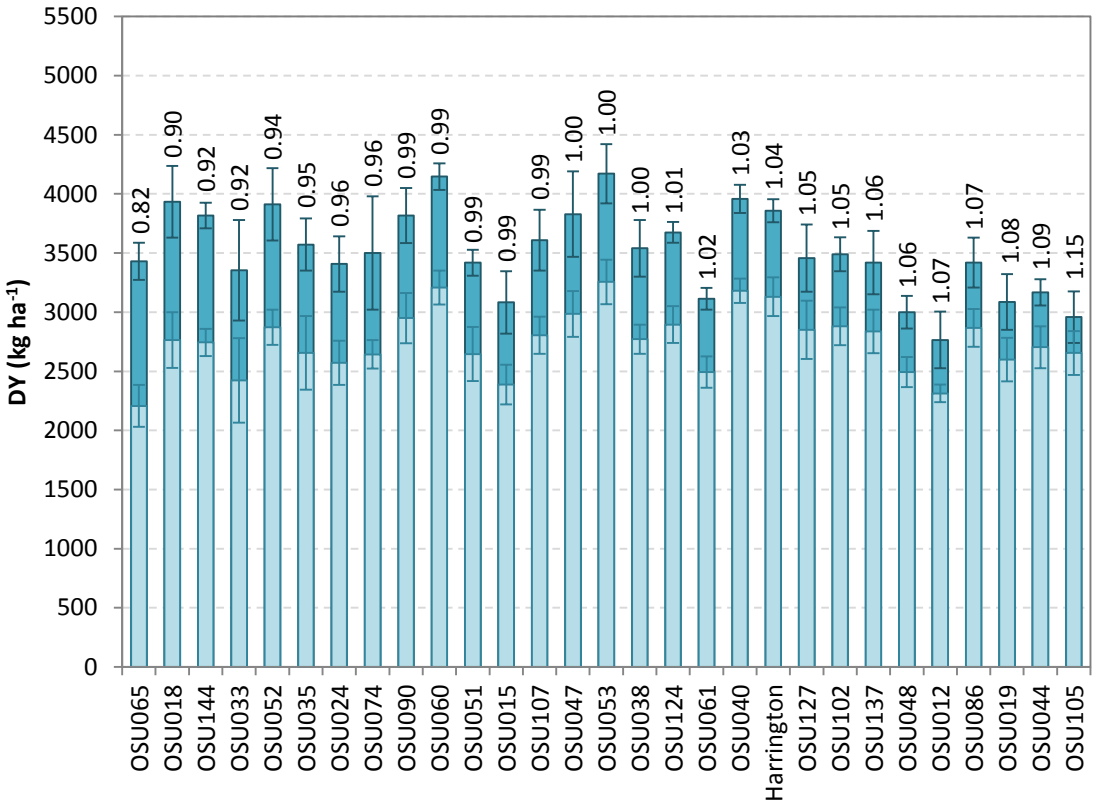

Figure 2.13. Overall dry yield values accounted for each RCSLs and cv. Harrington in the well-watered treatment (dark blue bars) and water stress (light blue bars) across field trials. Drought tolerance index (DTI) values are indicated on top of each bar for each genotype. Error bars represent the standard error of the mean.

In general, drought tolerant RCSLs were low yielding lines under favourable conditions (Fig. 2.14). The most drought tolerant lines had low yield potential but stable yield across water treatments. For example, yield values for OSU105, the most drought tolerant RCSL, were not significantly different across the water treatments, with mean values of 2958.3 kg ha^{-1} and 2654.9 kg ha^{-1} for WW and WS conditions respectively. However some tolerant RCSLs had high yield potential similar to cv. Harrington and maintained high yield values under water stress conditions. This is the case for OSU040 and OSU053 for which yield values under drought corresponded to 3180.5 kg ha^{-1} and 3255.7 kg ha^{-1}.

In contrast, drought susceptible RCSLs were generally high yielding under favourable conditions but water stress had a significant impact on their production. For example, OSU018 and OSU144 mean yields were 3933.9 kg ha^{-1} and 3816.8 kg ha^{-1} respectively for the WW condition but the mean yield production overall decreased around 29% with values of 2764.5 kg ha^{-1} and 2743.9 kg ha^{-1} for water deficit stress. DTI correlated negatively with heading date in WW and WS condition. Also, a negative correlation was found for biomass and dry yield production in WW.

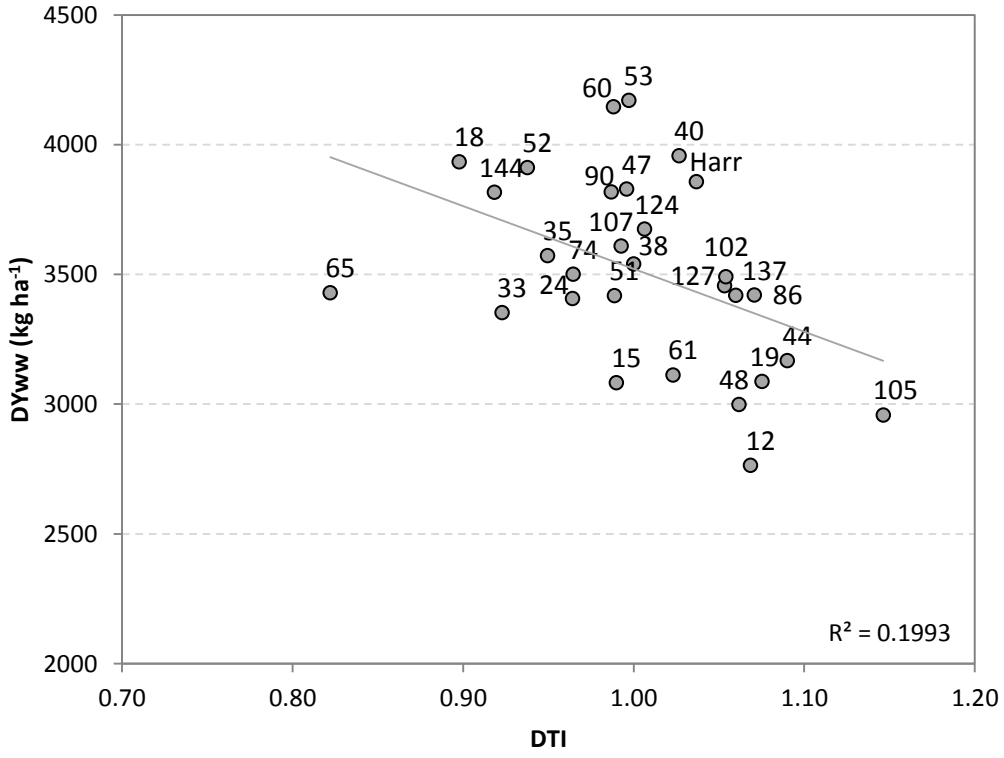

Figure 2.14. DTI values (x axes) plotted against genotypes potential yield (DYww, y axes). Data-points numeration correspond to the RCSLs (OSU) number; cv. Harrington shown as "Harr". Fitted line by linear regression ($P<0.05$)

The drought tolerance index was useful to classify the genotypes according to their yield stability but it has some limitations to be used as selection criteria for RCSLs that potentially could contribute allelic variation to enhance the elite cultivar drought tolerance since, in general, selection for high DTI values would tend to reduce potential yield under favourable conditions (Fig. 2.14). The mechanisms behind the drought tolerance phenotype of these lines might be different. Early flowering lines such as OSU105 and OSU019 could have escaped water stress by shortening their days to heading; however this phenotype had a negative effect on yield production and these lines had low but stable yields across water treatments, hence the high DTI value.

The phenotypic differences between drought tolerant and susceptible RCSLs with high yield potential under favourable conditions would define the key phenes and ultimately, the chromosome regions, that potentially could contribute allelic variation for crop improvement under water deficit conditions.

ii. Yield components genotype by environment interaction

The AMMI model was used to estimate the genotype by environment (GE) interaction effect on yield and yield components (Gauch, 2006). This method has been widely used to assess adaptation and yield stability in crops (Rodriguez *et al.*, 2008; De Vita *et al.*, 2010; Mohammadi and Amri, 2013). It is essentially another way to measure stability in the lines performance across environments, but assessing also the genotypes yield potential. For this test, the three water treatments were considered in combination with the field growing season to define six test environments: Full irrigated, partially irrigated and drought in 2013 and 2014 (named as FI13, PI13, DR13, FI14, PI14 and DR14 respectively).

The AMMI analysis of variance for dry yield of the 29 genotypes studied in 6 test environments showed that the genotypes and the environments had a highly significant effect ($P<0.001$) explaining 17.6% and 44.1% of the model sum of squares respectively (Table 2.9). The GE interaction accounted for 9.3% sum of squares and was not significant for the experimental conditions ($P=0.0682$). Therefore the environment was the main source of variation for dry yield and the magnitude of the GE interaction effect on this trait was minor compared to the effect of the genotype overall. Nevertheless, the AMMI1 biplot for dry yield (Fig. 2.15), which combines the potential yield of the genotypes with the IPCA1 score as a measure of stability (Vargas *et al.*, 1999), was used to assess the trend observed in the stability of the RCSLs referred to the genotype mean performance across environments and to define contrasting responses. Lines such as OSU053 and OSU060 seemed to be high yielding lines with reasonably stable values across the six environments, especially OSU060. In contrast, genotypes

such as OSU144 and OSU012 tended to be the most responsive, being high yielding and low yielding, respectively, but with quite unstable yield across the environments tested.

Table 2.9. AMMI analysis of Dry yield (DY) and thousand grain weight (TGW) of RCSLs and Harrington across six environments (treatment/year combination)

Source of variation	d.f.	DY			TGW		
		s.s.	s.s %[1]	P-value	s.s.	s.s %[1]	P-value
Model	695	364916846			8925		
Genotypes (G)	28	64299273	17.62	<0.001	2930	32.83	<0.001
Environments (E)	5	161011460	44.12	<0.001	2612	29.27	<0.001
Reps within E	18	5202435	1.43	0.1035	348	3.90	<0.001
Interactions (GE)	140	33908173	9.29	0.0682	930	10.42	<0.001
IPCA1	32	13549450	*39.96*	<0.001	394	*42.37*	<0.001
IPCA2	30	8248377	*24.33*	0.0895	226	*24.30*	0.0064
Residual	78	12110346	*35.72*	0.9141	310	*33.33*	0.5958
Error	504	100495504	27.54		2106	23.60	

[1] Percentage of model sum of squares for genotypes (G), environments (E) and the interaction (GE); percentage of GE sum of squares for IPCAs in italics.

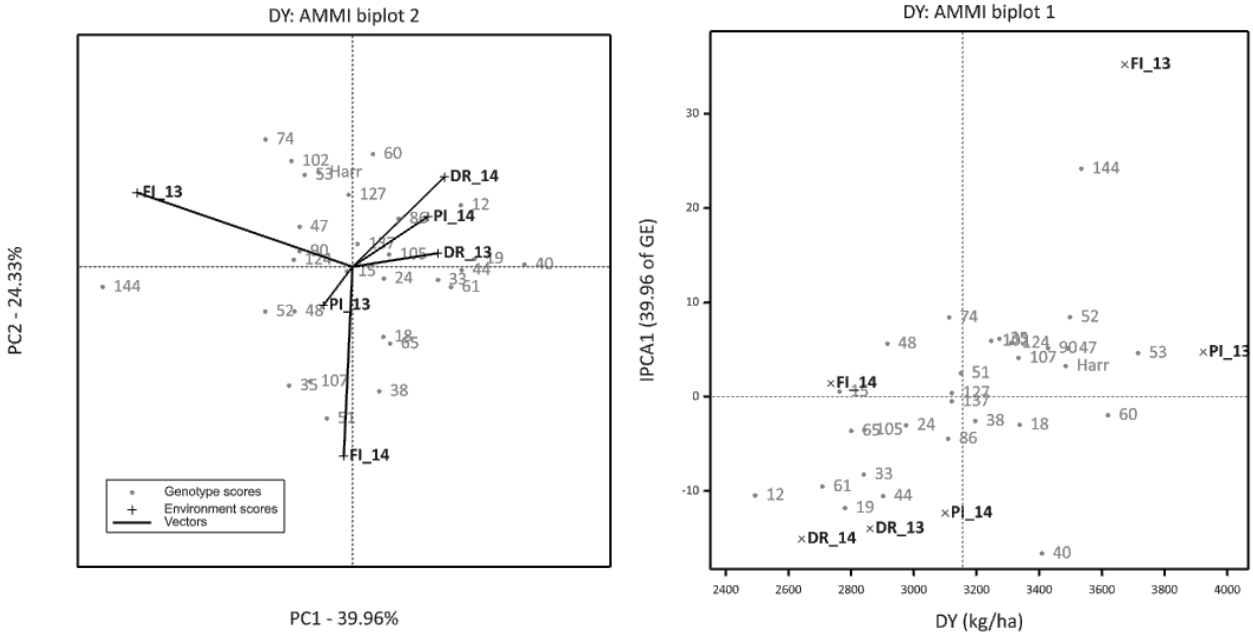

Figure 2.15. AMMI2 (left) and AMMI1 (right) biplots for dry yield (DY) with the RCSLs and cv. Harrington evaluated in field trials over three water treatments (FI, PI, DR) and two years (13, 14) (6 environments or year/treatment combinations). Genotypes score in grey numbered according to the RCSLs (OSU) number. Environments score in bold capital letters. Lines correspond to the environment vectors in the AMMI2 biplot. AMMI2 biplot represents the IPCA1 (x axes) versus IPCA2 (y axes) showing the magnitude of interaction of each genotype and environment. AMMI1 biplot relates the overall yield mean of each genotype (x axes) and the IPCA1 score (y axes) used as a measure of yield stability.

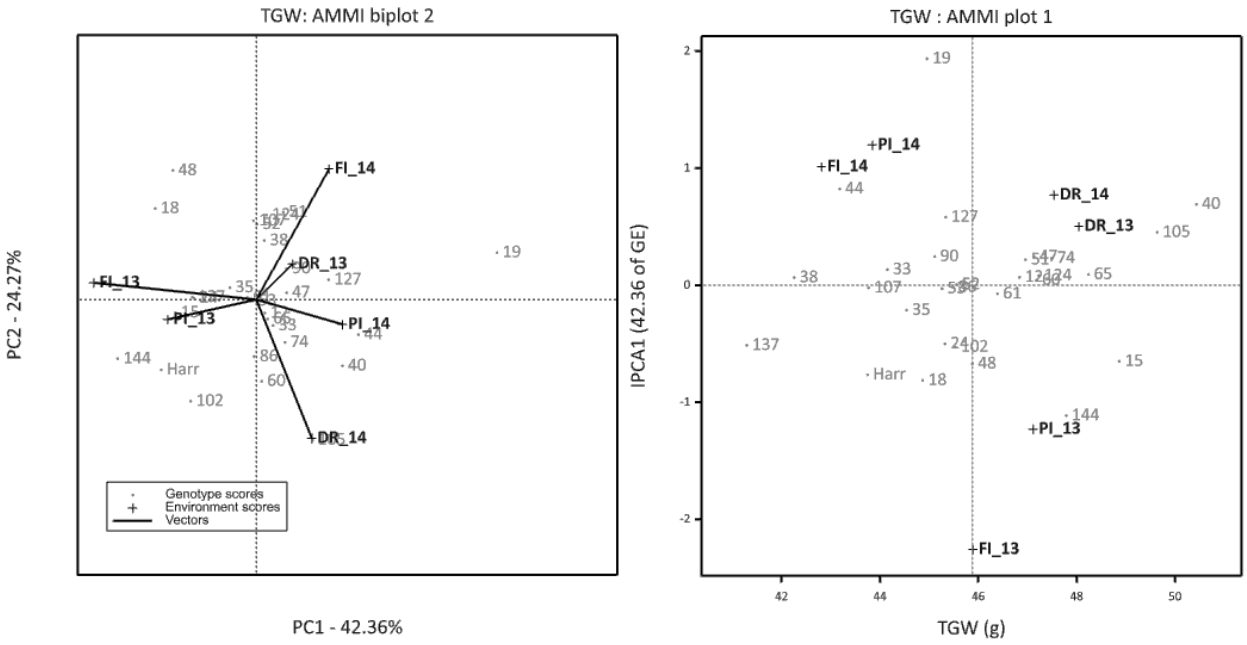

Figure 2.16. AMMI2 (left) and AMMI1 (right) biplots for thousand grain weight (TGW) with the RCSLs and cv. Harrington evaluated in field trials over three water treatments (FI, PI, DR) and two years (13, 14) (6 environments or year/treatment combinations). Genotypes score (in grey) numbered according to the RCSLs (OSU) number. Environments score in bold capital letters. Lines correspond to the environment vectors in the AMMI2 biplot. AMMI2 biplot represents the IPCA1 (x axes) versus IPCA2 (y axes) showing the magnitude of interaction of each genotype and environment. AMMI1 biplot relates the overall TGW mean of each genotype (x axes) and the IPCA1 score (y axes) used as a measure of yield stability.

AMMI test was also conducted for TGW. In this case the analysis showed that the environments (E), genotypes (G) and the GE interaction had highly significant effect (P<0.001) and accounted for 29.3%, 32.8% and 10.4% of the model sum of squares respectively (Table 2.9). The weight of the genotype on the sum of squares was larger than that for environments, indicating substantial genetic variation. In addition, the results showed that there were noticeable differences in the genotypes stability across testing environments. GE interaction was further partitioned by principal component analysis. The AMMI analysis showed that the first two principal components of the interaction, IPCA1 and IPCA2, explained 66.7% of the GE sum of squares, leaving a non-significant residual term. The IPCA1 accounted for 42.4% of GE interaction and the IPCA2 for 24.3%.

The IPCA scores are defined for environment and genotypes. The IPCA scores of the genotypes are an indication of adaptability over environments (Vargas *et al.*, 1999; Lacaze *et al.*, 2009; De Vita *et al.*, 2010). The larger the IPCA scores of a genotype, the more adapted is the genotype to specific environments (those with IPCA values of the same sign). In contrast, genotypes with IPCA scores close to zero are more stable or widely adapted to the tested environments. In

figure 2.16 (left) the IPCA1 and IPCA2 scores for the genotypes and the environments are represented (AMMI2 biplot). Genotypes OSU048, OSU018, OSU105, OSU144 or OSU018 are examples of responsive genotypes being the best or the worst adapted for any or several environments. For example, OSU015, OSU144 and OSU137 were well adapted to the irrigated environments in 2013 (FI13 and PI13). In contrast, OSU019, OSU127 and OSU090 were better adapted to the droughted environment the same growing season (DR13). The elite barley, cv. Harrington, responded well to the irrigated environments in 2013 (FI13 and PI13), however the irrigated conditions in 2014 (PI14 and FI14) and the water deficit environment in 2013 (DR13) were not as favourable. Genotype OSU044 appeared to be the best line when averaged for the droughted environments (DR13 and DR14), OSU048 for the full irrigated (FI13 and FI14) and OSU060 for the partially irrigated (PI13 and PI14) environments.

The AMMI1 biplot (Fig. 2.16, right) take the main effect of the genotype into account to test for stability (IPCA1). By looking at the scatter of the genotypes in this biplot we can observe that OSU019 and OSU144 were contrasting genotypes for IPCA1, and OSU137 and OSU040 were extreme lines for genotype main effect. The first two RCSLs were unstable in the GE interaction, showing specific adaptation to the irrigated environments in 2013 (OSU144) and 2014 (OSU019). On the other hand, OSU137 seemed to be poorest in performance, with the lowest mean across environments and OSU040 was the best in performance, with the largest genotypic mean across environments and specially favoured by the droughted environments (DR13 and DR14). Interestingly, RCSLs such as OSU060 and OSU053 not only had genotype main performances larger than cv. Harrington, but were broadly adapted to the environments tested showing more stability in TGW overall (IPCA1 close to zero). Hence, these two lines could be an interesting source of allelic variation for the crop as they conserve the agronomic performance of an elite variety showing significantly improved TGW and adding stability to the crop performance. The correlation analysis revealed that TGW IPCA1 correlated negatively with biomass yield and heading date.

2.4 Discussion

For the present study, a set of 29 barley Recombinant Chromosome Substitution Lines (RCSLs) harbouring introgressions of a drought tolerant accession of *Hordeum vulgare* ssp. *spontaneum* (Caesarea 26-24) in the same elite cultivated barley genetic background (cv. Harrington) have been evaluated under different water regimes during the 2013 and 2014 growing seasons at The James Hutton Institute, Dundee. The two field trials were carried out in rain-out shelters to evaluate the impact of water deficit on the RCSLs phenotype for morphological, developmental and agronomic traits. The main goal of this work was to determine the effect of the exotic genome on the performance of the cultivated barley and define whether it contributes new allelic variation that enhances performance in less favourable environmental conditions. In the following sections, the main variations found among genotypes, treatments, years and their interactions are discussed.

2.4.1 Drought tolerance phenotyping in field conditions

Phenotyping for drought tolerance is a challenging task, not only because of the variation of the drought responses and the multigenic nature of the trait, but also because of the difficulties in defining the intensity of the stress which ultimately will lead to different levels of response (Araus *et al.*, 2002; Passioura, 2007; Tuberosa, 2012).

2.4.1.1 Rain-out shelter as a semi-controlled set-up to assess water deficit in the field

Rain-out shelters are principally used to avoid the effect of natural precipitation on drought field evaluations of crops (Day *et al.*, 1987; González *et al.*, 1999; Ober *et al.*, 2004; Yue *et al.*, 2006; Hoffmann *et al.*, 2011) and natural grassland populations (Lucas *et al.,* 2008; Signarbieux and Feller, 2012). Despite some experimental constraints, mainly related to the limited extension of the trials and plot sizes, they offer an important advantage for conducting field experiments under semi-controlled environmental conditions. Equally important, due to the microclimatic conditions inside the tunnel, both control (non-stressed) and stress plots are protected by the shelter maintaining similar experimental conditions throughout as remarked in Blum (2011).

The reduction in soil moisture and overall diminished agronomic performance of the RCSLs and cv. Harrington evidences the success of the rain-out shelter's experimental approach for imposing moderate drought in the field in 2013 and 2014. The differences in yield across the water treatments can be associated with variation in available water in the soil profiles, particularly in the droughted plots where the progressive decrease in moisture throughout the experiment significantly limited plant growth and yield production in both years. However,

variations in climatic conditions and the management of the water treatments in each trial might explain some of the significant differences found across growing seasons as stated below.

2.4.1.2 Seasonal variations shaped the stress conditions across growing seasons

Overall, 2013 was better in performance for the three water treatments compared to 2014. Greater differences between treatments were also observed, between years. Yield in 2013 was reduced by 27% compared to 15% in 2014. Important differences in the environmental conditions across the years might explain the observed variation in the field. For example, in 2013, the combination of reduced soil matric potential due to water stress and increased mechanical impedance estimated in soil samples taken at 40 cm depth in the field site suggests that plants under drought found penetration resistances >2MPa at a low matric potential (<-250 KPa). In a review on the soil physical constraints limiting root elongation in arable soils, Bengough *et al.* (2011) concluded that root elongation slows down in drying soils due to a combination of water stress and mechanical impedance, and that a penetrometer resistance of 2 MPa is enough to reduce root elongation to less than half of its unimpeded rate. Therefore, it could be that plants found it more difficult to explore deep soil water resources in 2013 due to the subsoil stress conditions. Nevertheless, the decrease in soil moisture in deep soil layers in the drought plots during heading and flowering period in 2013 suggests that stressed plants were capable of using water from subsoil during the most susceptible growth stages under water deficit. The same trend was observed in 2014 but with no limiting physical constraints for root elongation in deep soil layers. The measures here are based on means rather than individual plots and no estimation on the use of soil moisture at the genotype level was done; however, it could be that differences in phenology and vigour across the RCSLs had an effect on the soil water available for the different genotypes at key developmental stages. Inostroza *et al.* (2011) found segregation for traits defining seedling vigour in the RCSLs. Although high vigour was not necessarily associated with drought tolerance, it is a characteristic that is linked to improved water use efficiency (WUE), likewise phenological adjustment or earliness (Araus *et al.*, 2003). RCSLs with increased vigour might have improved the exploration of the soil to capture resources when the conditions were restrictive for plant growth due to a higher proportion of roots mass per shoot. Additionally, early maturity lines could have reduced the evaporative loss of water from the soil surface ensuring more growth and transpiration when vapour pressure deficit was small.

In contrast, although the soil physical conditions were presumably better for root soil exploration in 2014 than in 2013, 2014 was generally poor in performance for the RCSLs especially under irrigation. The water stress had a significantly negative effect on plant growth and yield, but the differences across the water treatments were not as marked as in 2013. The

diminished agronomic performance was especially notable in the irrigated plots for which average yield was 25.5% and 20.9% lower than in 2013 for the full and the partial irrigated treatments respectively. The difference for the drought plots was 7.6%.

It appears that the environmental conditions in 2014 were less suitable for determining the impact of late drought in the field trial, not because the droughted plots did not limit their growth and production due to the stress imposed, but because of the relatively low yield values reached in the watered plots. It could be that the irrigation management in 2014 field trial or variation in other environmental conditions across the years imposed some limitation for achieving high yield potential in the RCSLs. Besides, the accumulated rainfall prior to the beginning of the experiment was greater in 2014 than in 2013, as there were more rainy days and milder temperatures throughout the growing season. This could have had an effect on the increased groundwater moisture levels as observed with soil moisture measures, which could have resulted in an excess of water in the irrigated plots leading to waterlogging and therefore a negative impact on crop production under irrigation.

Additionally, other seasonal factors which varied across years might have determined the differences in development of the crop across the trials. The 2014 growing season was significantly shorter than in 2013: plants reached heading 5 days earlier on average within a period of 10 days (18 days in 2013). The higher soil temperature at the time of sowing and seedling establishment in 2014 could have accelerated the seed germination by 3–4 days compared to 2013 as indicated in the guide of *Barley growth and development* (Fettell *et al.*, 2010). Furthermore, a shortened vegetative growth period would explain the significant reduction in number of tillers for all the genotypes in 2014, resulting in fewer spikes harvested and a lower final yield.

2.4.2 Wild barley introgressed chromosome regions affected RCSLs performance

The RCSLs phenotype was characterized for fourteen morphological, developmental and agronomic traits. The effect of year, treatment, genotype and their interaction was evaluated by fitting a three factorial mixed model to the data. Besides the differences across growing seasons and the treatments across trials, the analysis revealed highly significant differences among the RCSLs for all the traits and, in many cases, its interaction with water treatment suggesting not only a strong effect of the wild barley introgressions in the phenotype, but also differences in the response to the water treatment. In order to determine the effect of the exotic genome introgressions on the performance of the lines, a multi-comparison test of the estimates defining the RCSLs phenotype was performed using the cv. Harrington values as control. This test was

carried out for all the traits measured and it allowed groups of lines for which phenotype was improved or diminished potentially as a consequence of the exotic introgressions to be identified. This following section discusses the results obtained for each group of traits.

2.4.2.1 Morphological traits

Barley breeding has led to the development of semi-dwarf varieties with an enhanced lodging resistance and increased harvest index (Bezant *et al.*, 1996), which has improved the harvesting (short and stiff erect plants). However, water deficit can limit plant growth considerably and affect negatively yield production in cultivated barley due to the reduction in plant height (Shakhatreh *et al.*, 2001). In fact, although dwarfing genes help to maximize yield of cereal crops in favourable environments, it has been associated with a decline of seedling vigour, and consequently crop establishment, in drought-prone environments, contributing to yield reduction under stress as has been reported for wheat (Rosyara *et al.*, 2009). In addition, in a review on drought tolerance, Cattivelli *et al.* (2008) highlighted the importance of single genes related with traits as plant height in the adaptation to drought-prone environments and their major effect on yield despite the quantitative nature of drought tolerance.

In the study by Badr *et al.* (2000), morphological traits such as plant height, spike length and width, awns length, etc. were found to have superior capacity to discriminate wild barley from the cultivated forms. Different authors have found these traits to contribute significantly in the offspring derived from backcrosses between an elite cultivar and a wild donor (Pillen, *et al.*, 2003; Li *et al.*, 2005, 2006; Gyenis *et al.*, 2007; Inostroza *et al.*, 2009; Schmalenbach *et. al.*, 2009b; Kalladan *et al.*, 2013). Assessing the morphological adaptation in response to stress conditions will improve our understanding of how the plant readjusts its source and sink relations that ultimately will affect yield in less favourable conditions and define the drought tolerant phenotype for each environment.

i. Plant height

Considerable variation for height was found between RCSLs and across treatments. The elite parent height was close to the optimum range defined for modern cereal cultivars (between 70 and 100 cm; Richards, 1992). However cv. Harrington was considerably shorter than the majority of the RCSLs with HEI values that were 13 cm and 9.8 cm larger on average, (11.6% and 8.8%) in 2013 and 2014 respectively. Similar results were observed in other wide crosses. For example, Schmalenbach *et al.* (2009b) found that the wild barley alleles (from the 'ISR 42-8' accession) increased plant height by up to 15.9 cm (19.7%) compared to the cultivated barley (cv. Scarlett) in a set of 39 lines from a BC_2DH population (von Korff *et al.*, 2006). In this

study, most of the exotic introgressions associated with the trait were found to increase plant height (six out of seven). In contrast, Inostroza *et al.* (2009) reported that the wild barley introgression decreased plant height in an association study using 80 RCSLs (six out of eleven). In this work, reduction in plant height was considered a favourable effect of the donor parent since it was correlated with higher grain yield. The effect of the genotypic variation found for plant height on yield production will be discussed in section 2.4.3.

Regarding the effect of the treatment, plant growth was significantly limited as a consequence of the water deficit, resulting in diminished yields overall. Average collar height was 11.7% shorter in 2013 under drought (9.7% in 2014). This effect has been broadly reported in similar studies, for example, Inostroza *et al.* (2009) reported a 16% decrease in plant height in the Mediterranean Chilean environments in response to the water deficit. They also noted a decrease of 45% in height in a field experiment in contrasting locations in Oregon.

Interestingly, most of the significantly taller genotypes seem to resist lodging. For example, OSU033 reached a maximum height of 147 cm in both trials, but was not prone to lodging. In contrast, severe lodging was observed in OSU065 which was almost as tall as OSU033 (137 cm and 140.7 cm in 2013 and 2014). These two lines, however, were low yielding over all; it could be that OSU065 was affected by the sensitivity to lodge in performance more than OSU033. In contrast, other significantly tall lines such as OSU040 (133.7 cm and 132.7 cm maximum height in 2013 and 2014) maintained yields similar to cv. Harrington. However, the wind protection of the rain-out shelter might have accentuated these differences. None of the previous RCSLs evaluations in field conditions have investigated lodging resistance in this population nor its effects on yield, nevertheless, further studies could provide valuable information to understanding the nature of this resistance and potentially identify chromosome regions conferring the tolerance.

ii. Peduncle characteristics

Water stress can inhibit peduncle growth in cereals, impeding, in some cases, the spikes to emerge out of the boot leaf. The results in this study confirm that general growth limitation in water stressed plants resulted in the significant reduction in the length of the peduncle (PdL and PdE). As with plant height, there was a strong effect of genotype on the phenotypic variation observed and most of the RCSLs had significantly longer peduncles than cv. Harrington. Several authors have shown that long peduncles are mainly seen in wild barleys since the semi-dwarfing genes introduced in modern cultivars seem to have major effect on stem length and so, peduncle length (Acevedo *et al.*, 1991; Borrell *et al.*, 1993; Forster *et al.*, 2004). Studies have shown that these traits are important to barleys' photosynthesis capacity and harbour the

majority of reserve carbohydrates storage that will be remobilised during grain filling (Daniels *et al.*, 1982; Ehdaie *et al.*, 2006; Blum, 2011). Therefore, length of the peduncle is considered a good indicator of yield capacity in low-rainfall environments (Acevedo *et al.*, 1991; Lakew *et al.*, 2011; Mohammadi and Karimizadeh, 2014). As with plant height, the exotic introgressions in the RCSLs appear to contribute to enhance the length of the peduncle of the elite parent. Even under water stress the average length of the peduncle in the RCSLs (32.9 cm in 2013 and 29.3 cm in 2014) was greater than the mean value for cv. Harrington under favourable conditions (31.6 cm in 2013 and 28.9 cm in 2014). Potentially, this could mean an advantage for grain yield under water stress in the RCSLs. Correlation of this secondary trait with grain yield will be discussed in section 2.4.3. Nevertheless, it has been shown by different authors that the ability of the plant to remobilise the stem reserves for grain growth under water limited conditions is more important than the absolute stored stem reserves accumulated (Borrell *et al.*, 1993; Przulj and Momcilovic, 2001; Bazargani *et al.*, 2011; Powell *et al.*, 2012).

iii. Spike and seeds characteristics

The phenotypic variation found for spike length and seed morphological characteristics such as seed length, width and area in the RCSLs was also highly determined by the genotype. These traits were found to have superior capacity to discriminate wild from cultivated barleys (Badr *et al.*, 2000; Gyenis *et al.*, 2007). Long ears and narrow and long seeds are commonly found in wild barleys. Of the group of sixteen RCSLs with significantly longer spikes, two lines (OSU015 and OSU044) had considerably longer and narrower seeds than the cultivated barley, showing a phenotype more similar to the wild parent. In addition, OSU015 showed some degree of awn retention and therefore reduced grain threshability compared to cv. Harrington and the other RCSLs. This unattractive trait for cultivated barley may be useful in subsistence agriculture situations where crops are at risk of high winds or hail damage as observed by Matus *et al.* (2003). In this initial screening of the RCSLs, the authors also reported other domestication-related characteristics such as seed shattering. This important seed dispersal mechanism in wild barley, but undesirable for the cultivated forms, has been observed in other wide crosses with a wild barley (Gyenis *et al.* 2007). The severe seed shattering observed in OSU012 in the present work was probably the reason for the lower overall yield values observed for this line harvesting.

Five of the sixteen RCSLs with significantly longer spikes have wider seeds with an increased seed area compared to the cv. Harrington (OSU040, OSU053, OSU065, OSU105 and OSU144). It may be that these lines segregate for the dry matter accumulation in the form of starch and nitrogen during the grain-filling phase post anthesis. Actually, in the study by Kalladan *et al.* (2013) on a BC_3-double haploid population obtained from a cultivated barley (cv. Breda) and a

wild barley accession (HS584), seed width was found to be positively correlated with starch content and negatively associated with nitrogen content. In addition, as reviewed in Coventry *et al.* (2003), it could be that segregation of chromosome regions harbouring genes associated with photoperiod and vernalisation response in barley might have influenced indirectly the size of the grain by defining the duration of the pre-anthesis period and predisposing the post-anthesis growth to different environmental conditions. The relationship found between seed characteristics and yield and yield components such as TGW will be discussed in section 2.4.3.

Interestingly, the RCSLs were found to segregate for waxy non-glaucous (glossy) spike phenotype since five lines (OSU012, OSU047, OSU060, OSU074 and OSU090) showed perceptible glossy spikes. Spike glossiness is a highly heritable character that has not been reported before in the RCSLs. Nevertheless, this phenotype has been frequently observed in wild barleys (Chen *et al.*, 2004; Nice *et al.*, 2016) and it is associated with a reduced accumulation of waxes leading to a bright-green appearance of the spike. Surprisingly, although this phenotype might relate to increased permeability of the cuticle to water loss (Zhang *et al.*, 2015), glossy genotypes did not show large variability in their grain weight as compared to the elite parent. Further investigations would be needed to assess the association of wax-less spikes with water stress tolerance and/or the desiccation of the spikes. It could be that in the experimental conditions of the present study, the stress conditions were not severe enough to cause large evaporative losses from the spike and that actually a reduced reflectance in the photosynthetic active radiation regions (PAR: 400-700nm) could have favoured increased net photosynthetic rate of the glossy spikes as compared to the non-glossy genotypes, which reflectance within this spectral range would be accentuated (Shepherd and Griffiths, 2006). This effect has been reported in non-glaucous wheat genotypes as compared to glaucous ones (Richards *et al.*, 1986). However, this does not seem very likely since it would only constitute an advantage in non-saturating light levels and also other non-glossy genotypes showed grain stability across environments. Therefore, other aspects of plant development may contribute to these differences. Despite the results obtained in this regard are not conclusive, the segregation observed for glossy spike makes this set of lines a very useful genetic resource not only to find candidate genes associated with this phenotype, but also to elucidate the protective role of waxes under water deficit to improve transpiration efficiency and control stomatal conductance.

2.4.2.2 Developmental traits

Plants show a wide range of responses to cope with abiotic stresses such as drought. Due to their sessile nature, their developmental plasticity is important in order to sustain yield under and survive stress conditions.

For instance, regulation in source–sink relationships under stress can modulate the impact of drought on plant fitness. In crop plants, the limitation in tiller number under drought could lead to an increase in grain mass per inflorescence (Blum, 2011). On the contrary, heading date is a key developmental trait playing an important role in the adaptation of cereal crops to variable environments especially in low rainfall conditions where earliness can be an advantage to avoid drought stress during the grain filling period (Araus *et al.*, 2008).

i. Tiller number

As expected, total tiller number was adversely affected by drought stress treatments in both years as a consequence of limited growth. According to Blum (2011), reduced tillering at the vegetative stage due to water scarcity in the topsoil can actually enhance deeper root development in grain crops. The phenotypic variation observed in the RCSLs tillering was highly influenced by the genotype but there was no differential response in the interaction with the treatment. Since barley nodal root systems arise from basal nodes of the main shoot and tillers, it could be that the genotypic differences found for tillering in the RCSLs correlate with genotypic differentiation in the development of root system across the RCSLs. This phenomenon was observed by Naz *et al.* (2012, 2014) in the wild barley introgression library S42ILs, derived from the initial cross cv. Scarlett × ISR 42-8. The authors of this work not only found segregation of root traits such as root length, root volume, and root dry weight but also positive relationship among these traits and change in tiller numbers. It could be that the two lines with significantly more tillers than cv. Harrington (OSU047 and OSU127) showed a more extensive root system which had a positive putative effect on shoot development in the water deficit treatment.

ii. Days to heading

The range of variation found for days to heading was essentially determined by the year and the genotype. In 2014, days to heading was significantly earlier than in 2013 with an average of 59.2 DAS to reach heading date compared to 64.0 DAS in 2013. Also, plants reached heading within a shorter period of time in 2014 (11 days) than in 2013 (18 days). The comparative analysis of the phenotypic variability found in the RCSLs with cv. Harrington revealed that fourteen RCSLs were significantly early in ear emergence whereas four lines were significantly late. Differences due to the water treatment were found only in 2013, where the fully irrigated plots were slightly later than the partial irrigated and the droughted plots.

Days to heading is a highly heritable trait determined by vernalisation requirement, photoperiod response and earliness *per se* genes in barley (Cuesta-Marcos *et al.*, 2008). In addition,

flowering time is also modulated by factors that will adjust the crop phenology to ensure an optimum seed set in specific environmental conditions. Temperature and photoperiod are the main seasonal cues driving this response (Karsai *et al.*, 2013). Since the photoperiod follows predictable patterns from year to year and in the present study, both field trials were sown around the same date (15th and 17th April in 2013 and 2014 respectively), photoperiod was assumed to have little impact on defining the optimum time for ear emergence. However, the variation in temperature across the seasons, with higher temperatures in 2014 may have accelerated the time to reach heading as well as the duration of the transition from vegetative to reproductive growth in the 2014 compared to 2013.

In addition to that, Karsai *et al.* (2004) showed that the spring habit phenotype of the cv. Harrington, with no vernalisation requirement and little response to photoperiod duration, is in contrast with that of the RCSLs wild parent, Caesarea 26-24, which is closer to the winter habit phenotype (responsive to long days and vernalisation). This would explain the significant variation found in the RCSLs heading date, with significantly early and late lines when compared to cv. Harrington. Surprisingly, the segregation of chromosome regions from the wild barley accession in a group of 80 of these RCSLs did not show big differences in flowering time in field evaluations carried out in Mediterranean environments in Chile (del Pozo *et al.*, 2012). According to this work, earliness was not an important mechanism determining drought response in the RCSLs. The impact of days to heading on agronomic traits will be discussed in section 2.4.3.

2.4.2.3 Agronomic traits

i. Dry yield

Yield was very variable across years, genotypes, and treatment, all having significant effects. However, the difference in response of the RCSLs to the lack of water was only significant in 2013. As mentioned in section 2.4.1.2., the 2013 trial was not only better in overall performance for the different lines, but also for assessing the impact of drought across the RCSLs since the soil moisture stress was greater than in 2014 and plants were exposed to pronounced stress conditions during grain filling. Later heading date in 2013 would have also increased the intensity of the stress at this stage. Nevertheless, the lack of water in both field trials decreased the yield production significantly for all genotypes when compared to the well-watered condition as a consequence of the limitation in growth under stress.

The wide genotypic variation observed for dry yield in the RCSLs in this study was also observed in previous field experimental trials using different sets of the original RCSLs

population (Matus *et al.*, 2003). Eighteen RCSLs had significantly reduced yield whereas ten lines exhibited similar performance to cv. Harrington. In general, previous studies revealed an overall inferior performance of the RCSLs for yield and yield components compared to the elite parent (Matus *et al.*, 2003; del Pozo *et al.*, 2012). However, they showed the potential of the RCSLs to contribute favourable allelic variation not only for secondary traits associated with yield as the number of grains per ear, ear length and thousand grain weight (TGW) but also for improving yield stability and adaptability (Inostroza *et al.*, 2007, 2009). This will be expanded upon in section 2.4.4.

ii. Thousand Grain Weight

Thousand grain weight (TGW) had a wide, yet consistent, range of variation that was significantly determined by the genotype, the treatment and their interaction, but there were also significant differences across the growing seasons. The difference in TGW across years was essentially due to the reduced performance in the irrigated plots in 2014 compared to 2013, probably as a consequence of the excess of moisture in this trial as already discussed above. In fact, at the treatment level, it is quite noticeable that the overall estimates for the irrigated treatments were significantly smaller than those of the drought treatment. Plants under water stress had similar mean values across the trials, however in both irrigated treatments in 2014 and the full irrigation in 2013 the values were significantly reduced. It could be that the excess of moisture during grain filling delayed the whole-plant senescence leading to poorly filled grains and unused carbohydrates in the straw in irrigated conditions (Yang and Zhang, 2006) whereas the droughted plants remobilised the carbohydrates assimilated and stored in vegetative tissues before grain filling more efficiently (Asseng and Van Herwaarden, 2003). Moreover, the anatomical structure of the grain confers some protection from drought allowing the grain water potential and content to be relatively insensitive to water stress resulting in a continued deposition of dry matter (essentially starch and protein) in the grain even when drought causes severe wilting of the leaves (Barlow *et al.*, 1980; Brooks *et al.*, 1982). Therefore, even though stressed plants senesced earlier than watered plants and they limited their growth producing fewer tillers and so fewer heads and seeds, the seed weight in the drought plots for the experimental conditions did not decrease in comparison with the well-watered plots.

Additionally, the variation found at the genotypic level showed that most of the RCSLs had an improved phenotype compared to the recurrent parent and only two lines had significantly lower TGW. The positive transgressive segregation for TGW is evidence that the donor wild parent contains favourable alleles for this trait. This effect is commonly observed in hybrids obtained through intraspecific crosses using adapted cultivars, landraces and wild relatives (Veteläinen,

1994; Rieseberg *et al.*, 1999) and it was reported in Matus *et al.* (2003) not only for TGW but also for grains per ear and ear length.

2.4.3 Secondary traits affected yield and yield components

Numerous phenotypic correlations between morphological, developmental and agronomic traits were found for the well-watered (WW) and the water stress (WS) condition as well as for each trait across water regimes. In most of the cases, the direction of association and the level of significance of correlation among pairs of terms followed similar trends in each water treatment. Interesting differences are described below.

2.4.3.1 Morphological traits correlated with yield components

Increased plant height positively correlated with increased biomass production (r=0.42*** in WW and r=0.35*** in WS) had no effect on final yield production and explaining the significant negative correlation between plant height and harvest index regardless of the water treatment (r=-0.47*** in WW and r=-0.46*** in WS). The introduction of semi-dwarfing genes in modern cultivars has been associated with increased harvest index under favourable conditions (Hedden, 2003), although in less favourable arid environments, Shakhatreh *et al.* (2001) found significant positive correlation between plant height and final grain yield in different barley cultivars. However, in the present work plant height did not have an effect on yield under drought, probably due to the fact that the water stress imposed was not as severe as the one of the study mentioned. Similar results were found in the 137 RCSLs during one growing season in contrasting water regimes in a Mediterranean climate site in Chile (GCP project, unpublished data) where plant height was negatively correlated with harvest index (r=-0.29*** in Sta. Rosa irrigated; r=-0.24** in Sta. Rosa drought) but no correlation was found with grain yield. Nevertheless, other morphological traits seem to be relevant in shaping final yield in different water regimes across the RCSLs.

Longer peduncles were significantly correlated with greater TGW in controlled conditions (r=0.48***) and drought (r=0.29***), however the correlation of TGW with final yield (DY) was only significant under well irrigated conditions (r=0.42***) and the contribution of long peduncles to dry yield was highly significant in well-watered conditions (r=0.30***) whereas this relationship was still significant but not so strong under stress (r=0.16*).

According to Ehdaie *et al.* (2006, 2008), cereal crops under environmental constraints like drought, high temperature or low light, depend more on the remobilisation of stem reserves for grain filling than on current photo-assimilates; in contrast, under favourable conditions the

contribution of the pre-anthesis assimilate stem reserves to grain yield decreases and grain filling depends more on current photosynthesis in leaves and to some extent in spikes. Longer peduncles in barley have been proposed by Acevedo *et al.* (1991) as a potentially useful trait to select for in low-rainfall environments as they might store larger carbon assimilates that can be remobilised to fill the grain under adverse environmental conditions as suggested by Borrell *et al.* (1993) for wheat.

However, the associations found in the present study show a slightly different trend since the correlation between the length of the peduncle and grain weight and yield was greater in the irrigated treatment compared to the water limited plots. It could be that the remobilisation of WSC (water soluble carbohydrates) to the grain in genotypes with longer peduncles contributed positively and more significantly in defining grain weight (TGW) and yield (DY) in the irrigated plots whereas in the droughted treatment a substantial genotypic variation in the ability to remobilise stem reserves for grain growth was more important than the absolute WSC accumulated in the stem. Interestingly, del Pozo *et al.* (2012) did not find an increased mobilisation of WSC from the stem to the grains under terminal drought conditions in a set of RCSLs evaluated in field experiments in Chile, although they suggested an increased mobilisation of carbohydrate for spike and grain growth in a more favourable irrigated environment. Additionally, Mendez *et al.* (2011) showed important genotypic variations in the accumulation of stem soluble carbohydrates in a subset of 4 of these 24 RCSLs. The differences found in the accumulation of WSC in the RCSLs under drought was not associated with yield but with increased water stress tolerance due to the differential translocation of osmolytes associated with resistance to abiotic stresses as fructans.

2.4.3.2 Developmental traits correlated with yield components

Reviews on drought tolerance in cereal crops such as Araus *et al.* (2008) and Cattivelli *et al.* (2008) highlight the importance of early flowering as an escape mechanism to avoid water stress, particularly in environments where cereal crops are exposed to terminal drought stress having an impact on the reproductive stages of the crop. In barley, González *et al.* (1999) and Shakhatreh *et al.* (2001) found that earliness contributed to greater yields under terminal water stress in different barley cultivars and breeding lines whereas the correlation between phenology and yield was not significant under well-watered conditions. In contrast, other studies showed that early flowering was not a decisive trait to improve yield in barley grown under terminal drought conditions (Samarah *et al.*, 2009; Lakew *et al.*, 2011).

In the present study, final yield positively correlates with biomass yield, tiller number, harvest index and heading date regardless of the water treatment. These correlations were stronger in the well-watered conditions than under stress (r=0.88*** for BY, r=0.62*** for TILL, r=0.61*** for HI and r=0.51*** for HEA in WW; r=0.83*** for BY, r=0.51*** for TILL, r=0.52*** for HI and r=0.21** for HEA in WS). It seems that early flowering was not advantageous for the RCSLs yield production under drought; in fact it was detrimental despite the fact that late heading correlated negatively with tiller number under stress (r=-0.39***). This correlation was not found under irrigation. In barley, Mitchel *et al.* (1996) found that late maturing cultivars had a greater potential to extract soil moisture than the short-duration cultivars, especially at depth, which contributed to maintain higher crop growth rates and higher biomass production in field drought trials.

Grain weight contributed significantly to final yield under irrigation (r=0.42***) whereas it was not correlated under stress (r=0.12, ns). This suggests that, under the stress conditions imposed, plant phenology was not such a significant trait for defining yield as it has been shown in other studies for barley. Indeed, del Pozo *et al.* (2012) did not find phenology as an important trait to explain the RCSLs grain yield differences in a Mediterranean water limited environment. Furthermore, it could be that the plants were capable of adjusting the source–sink relationship to maintain grain production under stress, either by increasing the number of grains per spike in compensation for a decrease in fertile tillers or by increasing grain weight due to a decrease of number of grains per spike as suggested by Blum (2011).

The consistency in the results across field trials was reflected not only at the year level but also in regard to the water regime. The RCSLs tend to perform similarly across water treatments in the same site. This finding is supported by high autocorrelations for investigation of traits under the two contrasted water regimes using both trials data together for WW and WS. Traits like heading date and plant height had the highest correlations across treatments (r=0.98*** and r=0.90*** respectively) revealing the high heritability of these traits. Similar results were obtained in the field evaluation of the 137 RCSLs during one growing season in contrasting water regimes in Santa Rosa, Chile (GCP project, unpublished) where the highest correlation across treatments corresponded to plant height (r=0.55***); no data was collected on days to heading.

The lowest significant autocorrelations for the traits evaluated were found for biomass yield (r=0.59***) and dry yield (r=0.69***) and no correlation was found for tiller number across water treatments. Interestingly, the autocorrelation for grain yield was also found in the Chilean experiment (r=0.50***). Therefore, despite the significant variations found in this study in

regard to the impact of drought on yield across the RCSLs (defined in section 2.4.2.3.), it seems that genotypes performing well under favourable conditions tend to have relatively good yields under drought across different locations. This trend has been widely observed in barley and, as discussed by Tambussi *et al.* (2005) using a case study examining two barley cultivars across a wide range of environments, higher yield potential is an advantageous trait to increase yield in less favourable environments, especially under moderate water deficit conditions. However, under severe drought, low yield potential cultivars may perform better. The lack of correlation in number of tillers developed across water treatments reflects the plasticity of this trait in comparison with other yield components modulating final yield such as grains per spike or seed size. Tillering is highly influenced by the environment and trend has been widely documented for small grain cereal crops as discussed in Sadras and Slafer (2012).

2.4.4 Wild barley introgressions effect on yield stability across environments

As it has been already discussed, RCSLs with high yield potential seem to perform better across water treatments, however, the highly significant interaction between genotype and treatment for agronomic traits as dry yield (DY) and grain weight (TGW) revealed some genotypic differences in response to the water deficit. Two approaches were used to explore the variation found for these traits at the genotype by treatment level: the drought tolerance index (DTI) and the additive–main-effects–multiplicative interaction (AMMI) model.

2.4.4.1 Drought tolerance index (DTI)

The DTI defined groups of more tolerant and susceptible lines comparing the well-watered and the water stressed conditions. The recurrent parent was classified as tolerant for the experimental conditions; however, high DTI values (more drought tolerant lines) were not broadly associated with high yielding phenotypes. Similar results were reported by Lakew *et al.* (2011) in a study with barley introgression lines derived from crosses with two wild barley accessions and evaluated in five Mediterranean low-rainfall locations in different years. In this study, the majority of the lines classified as tolerant to drought were not necessarily good yielding lines in the stress environments, but stable. In addition, DTI was negatively correlated with final yield (r=-0.45*) and biomass (r=-0.69***) in the control conditions confirming the fact that low potential yield lines tend to perform badly under stress as it was discussed in the previous section. Also, the negative correlation found between DTI and days to heading in both water treatments (r=-0.53** in WW and r=-0.52** in WS) shows how earliness was detrimental for tolerance to drought for the experimental conditions. This was the only correlation found between DTI and the traits measured in the water stress treatment. Therefore, in the present

study, DTI was not a good index to select RCSLs better adapted to the stress conditions imposed in the field trials.

This result contrasts with the work by Inostroza *et al.* (2007). In the cited study, the authors found the DTI as a good parameter to select RCSLs with improved adaptation to water stress conditions. A positive correlation between the DTI and yield under drought was found using a set of 80 lines evaluated in one growing season (2004/05). Similarly, in a later study using a smaller set of RCSLs (24 lines), del Pozo *et al.* (2012) also found a significant correlation between the DTI (referred to as stress sensitivity index in this work) and grain yield, not only in the water stress environment but also under favourable conditions. Similar results were found using the data collected for the GCP project (unpublished). Interestingly, cv. Harrington was included within the group of tolerant lines in these studies (as in the present work) but some RCSLs showed an improved drought tolerant phenotype compared to the cultivar. Del Pozo *et al.* (2012) identified tolerant lines with high yield potential in the range of the recurrent parent but with increased $\Delta^{13}C$ in the grain, which is normally associated with improved transpiration efficiency in small grain cereals (Ferrio *et al.*, 2007). Unfortunately, the selection criteria for choosing the RCSLs evaluated in each of these experiments was different and no comparison could be made between the groups of tolerant/sensitive RCSLs identified in the cited publications and the present study. Nevertheless, some similarities were found when obtaining the DTI value for the 137 RCSLs in one growing season (GCP project, unpublished). Ten out of fifteen tolerant RCSLs in the present study scored a DTI value greater than 1 in the 2009 growing season in Santa Rosa, Chile. Despite the fact that in this case tolerance was positively correlated with yield under drought as in the other Chilean studies, it could be that these lines are more 'yield resilient' across different water deficit environments and it would be interesting to investigate the mechanisms underlying this response.

2.4.4.2 AMMI analysis

The AMMI model was found suitable to simplify the analysis of the significant differences found in the RCSLs agronomic performance as a consequence of the environmental conditions (year and water treatment effects). With this method the overall variation observed for yield and TGW was partitioned into genotype main effect (G), environment main effects (E) and genotype by environment interaction (GE). The biplots generated helped to integrate the information of the RCSLs yield potential and yield stability for a range of six environments (growing season/water treatment combination). This model has been regarded as a powerful analytical tool to investigate the GE interactions in yield multi-trials data (Gauch, 2006) and it has been applied in different studies not only to investigate the genotype-by-environment interaction in multi-trial data for different groups of barley genotypes (Nurminiemi *et al.*, 2002;

Rodriguez *et al.*, 2008), but also to assess the inheritance of the GE stability parameters (Emebiri and Moody, 2006).

The largest source of variation found for yield was the environment (44.1%), followed by the genotype (17.6%) and the GE (9.3%), although GE had a minor effect (not significant, p=0.0682). Thus, despite the large variation found for yield in response to the variation in the environmental conditions, the AMMI model confirmed that the genotypic differences drove the effect observed in yield rather than the interaction with the environment. Therefore, for the experimental conditions the RCSLs showed overall high-yield stability and, as discussed in section 2.4.3., high yield potential RCSLs tend to maximise the productivity in moderate water stress environments. This statement agrees with the fact that selection for greater yield potential in barley leads to higher production under moderate stress conditions (Tambussi *et al.*, 2005).

However, Inostroza *et al.* (2007, 2009) found important differences between RCSLs yield adaptability across six contrasting environments in Chile and USA using the Finlay and Wilkinson (1963) regression analysis approach. In this case, the locations considered differed greatly in annual rainfall defining at least two environments exposed to severe drought conditions. RCSLs with good yield in poor environments contrasted with RCSLs showing improved yield adaptability compared to the recurrent parent in favourable conditions. The authors found increased yield stability attributable to the wild barley alleles suggesting a functional role of the dehydrin genes on chromosome 6H that would need further investigation.

Even though the GE interaction was not found to be significant, the spread of the genotypes in the AMMI1 biplot was useful for selecting genotypes contrasting in their yield performance (high and low yield potential) and the trend in the adaptability pattern observed (specific or broad) for conducting a root phenotyping experiment that will be described in Chapter 4.

The AMMI analysis for TGW confirmed the substantial genetic variation found for this trait and discussed in section 2.4.2.3. In this case, genotypic variation (32.8%) was greater than the variation expressed by the environments (29.3%) and about 3-times the GE effect (10.4%); however, in this case the GE interaction was significant indicating differences in the stability of the genotypes across the environments evaluated. The variation found at the GE level was partitioned in two principal components (IPCA1 and IPCA2) that accounted for 66.6% of the variation. To investigate the divergences between the genotypes, the correlation between the IPCA axes and the genotype covariables were calculated. IPCA1 correlated negatively with heading date (r=-0.52**) and biomass yield (r=-0.44*); no correlation was found for IPCA2. This indicates that genotypes with higher IPCA1 scores tend to be earlier with less biomass,

while those with lower IPCA1 scores were generally later with larger biomass. The correlations found suggests that the plant phenology and morphology is well balanced by the resources and constraints of the environments which, at the same time, define the stability of yield components such as the grain weight. In other words, genotypes with an extreme phenology or biomass tend to have stronger GE interaction for TGW.

Despite the variation in response to the environment, what seems more remarkable is the broader adaptability of RCSLs for TGW compared to cv. Harrington. The elite parent showed better adaptability to the favourable irrigated conditions in 2013, which agrees with the fact that modern varieties are normally adapted to be more responsive to fully exploit nutrient and water resources when grown in optimum conditions as it has been described for wheat (De Vita *et al.*, 2010) and barley (Pswarayi *et al.*, 2008). However, the majority of the RCSLs outperformed the recurrent parent for this trait regardless of the water treatment, showing also a broader adaptability for this trait. Moreover, RCSLs such as OSU040, OSU060, OSU047 and OSU053 not only showed high nominal TGW values stable across environments but also had high yield potential values in the range of the elite barley. Similar results were found by Rodriguez *et al.* (2008) using a set of RILs derived from Sardinian barley landraces and modern varieties. The authors found that some RILs provided good yield levels and showed an intermediate GE interaction in comparison to modern varieties, suggesting that crosses between improved modern varieties and landraces adapted to Mediterranean environments may promote breeding material with environmental broad adaptability. In the present study, even if the environmental conditions were not extremely severe, there is evidence to conclude that the wild barley chromosome regions introgressed in the RCSLs may be beneficial in the improvement of the elite barley yield components performance and increased adaptability.

2.5 Conclusion

Improving drought tolerance is one of the major challenges in breeding programmes aiming to maintain high yield potential across a wider range of target environments which in some cases can experience some level of water deficit. However, drought tolerance responses are complex and vary depending on the stress level imposed. For this reason, it becomes essential to characterise the stress conditions in which plants are growing in drought field trials. In this chapter, the rain-out shelter proved to be a useful semi-controlled set-up for imposing moderate drought stress in the field during two growing seasons (2013 and 2014). Both years plant growth was significantly limited as a consequence of the water deficit, resulting in diminished yields overall. However, seasonal variations defined better environmental conditions in 2013 than in 2014 for reaching crop yield potential values in the non-stress treatment and measuring the impact of water stress on performance.

Field based studies of crops are more realistic and applicable from a breeding perspective than glasshouse approaches, however the interpretation of the response observed depends greatly on the analytic tools used to characterize the sources of variation affecting the phenotype (genotype, environment and the GE interaction). In the present work, the linear mixed model analysis was optimised to analyse the variation observed for each of the traits measured. This approach was suitable for determining the effect of the water treatment as well as the growing season and the genotype for each trait. However, since the fluctuation in the environmental conditions across years and treatments was large, the AMMI model analysis was found useful for simplifying the investigation of year and treatment effects on yield and yield components by partitioning the overall variation observed into genotype main effects (G), environment (E, identified as the combination of year and treatment) and their interaction (GE). These two approaches allowed the identification of lines with a significantly improved or diminished performance overall and enhanced adaptability compared to the recurrent parent. In contrast the other measure used defined as drought tolerance index (DTI), was not particularly meaningful to select genotypes that were better adapted to the stress conditions imposed in the field.

Despite the significant variation found in regard to the instabilities in the growing conditions, the wild barley chromosome introgressed regions had a strong effect on the traits measured. Even though the wild parent contributed with phenotypic traits lost during domestication such as awn retention (OSU015) and seed shattering (OSU012) as reported by Matus *et al.* (2003), the segregation found for other wild barley related traits such as glossy spike and the variation in lodging sensitivity are important agronomic traits that have not been mentioned in previous studies and would be worthy of further investigation.

In both field trials, none of the RCSLs outperformed the recurrent parent in terms of yield under the drought conditions imposed. In fact, high yield potential was found advantageous for performing well under moderate drought stress conditions. It seems that the stress imposed in the field was not very challenging for the elite cultivated barley. However, RCSLs accounting for similar yield values as the recurrent parent appear to have improved yield quality regardless of the water treatment. For example, OSU040 had greater TGW compared to cv. Harrington, the largest one in this group of lines and its final yield values were still in the range of the elite barley. Additionally, other high yield potential RCSLs such as OSU060, OSU047 and OSU053 showed a wider range of TGW than the recurrent parent which seems to be more specifically adapted to favourable environments. Interestingly, most of the RCSLs had greater TGW than cv. Harrington. This positive transgressive segregation evidences that the donor wild parent contains favourable alleles that not only enhance the performance of the trait but also its stability across environments. In Chapter 3, the chromosome regions associated with TGW and other relevant traits will be defined by association analysis using the phenotypic and genotypic data available for this set of RCSLs.

Assessing the effect of secondary traits on crop performance in a target environment helps to define key characteristics conferring adaptation to a certain stress level. In this case, the variation found for morphological and developmental traits seem to have affected the RCSLs final yield to some extent despite the fact that yield under drought was essentially determined by the genotype's yield potential. For moderate water stress conditions of the experiment, increased plant height and earliness negatively affected harvest index and final yield despite the fact that these traits have been generally considered relevant for adapting to water stress in drought-prone environments. In contrast, peduncle length seems to have a positive effect on the performance of the crop. Longer peduncles lead to improved TGW in the RCSLs, possibly as a consequence of an increased remobilisation of stem carbohydrates reserves to the grain. It may be that this wide genotypic variation found in shoot traits could be associated with variation in root traits, which is the focus of Chapter 4.

In summary, it can be concluded that the exotic genome contributes favourable alleles for improved adaptation and performance of the elite barley. Further investigations in sets of NILs derived from RCSLs such as OSU060, OSU040, OSU047 and OSU053 will be useful to elucidate the genetic basis of traits conferring adaptability to water stress environments as well as new allelic variation to improve the agronomic performance of the elite cultivated barley.

3 Marker–trait association analysis

Abstract

A marker–trait association analysis was conducted to localise quantitative trait loci (QTLs) controlling relevant agronomic and developmental traits in a set of twenty-eight Recombinant Chromosome Substitution Lines (RCSLs) representing the genome of the wild barley accession Caesarea 26-24 in the genetic background of the elite cv. Harrington.

The lines were genotyped with the 9K Infinium iSelect SNP chip for barley and evaluated in field trials over two growing seasons under different water regimes. The percentage of exotic genome introgressed in the RCSLs ranged from 3.7 to 26.6% with an average of 13.4% represented in about 6 introgressions per line. A REML-single locus analysis revealed 161 loci significantly affecting the phenotype at the marker main effect level or in the interaction with the treatment. The exotic genome was found to increase the trait performance in 55.3% of the associations observed. Some of these QTLs were verified in previous studies and also newly described QTLs were identified.

Main developmental loci were found to exert pleiotropic effects on yield related QTLs where the exotic alleles generally decreased crop yield performance compared to the elite barley alleles. However some positive associations were found for grain size and weight indicating that exotic genome could potentially be exploited for achieving genetic yield gains by improving the mobilisation and accumulation of assimilates in the developing grains during the post-anthesis period. Additionally, QTLs associated with traits conferring adaptation to drought-prone environments such as early flowering and plant cuticular waxes were identified and targeted for future investigations.

The results of the present study are encouraging for carrying out further association analysis using the 28 RCSLs in precise phenotyping studies and also for fine mapping studies in new backcrossed generations or near isogenic lines for targeted chromosome regions putatively associated with grain filling rate and drought adaptive traits.

3.1 Introduction

High-throughput genotyping technologies based on the detection of single nucleotide polymorphisms (SNPs) have become a highly automated and cost-efficient process that has considerably increased the resolution of genetic maps and the accuracy of QTL mapping studies (Rafalski, 2002b; Gupta *et.al.*, 2008).

The SNP-based genotyping technology has been recently used to uncover the genetic variation of quantitative traits in the three main sets of barley advanced backcross introgression lines in order to detect novel allelic variations for the crop improvement (Harrington × Caesarea (Matus et al. 2003 and this study); Scarlett × Israel ISR42-8 (von Korff *et al.*, 2004) and Haruna Nijo × H602 (Hori et al. 2005; described in detail in Chapter 1). Initially, the genotypic architecture of these three populations was established using SSRs (47 SSRs, 97 SSRs and 25 SSRs respectively), allowing the detection of several QTLs in association analysis for which the exotic genome was found to contribute favourable alleles to the elite barleys. The use of SNPs and the development of single assay high throughput SNP platforms, such as those developed by Illumina and described by Close *et al,*. (2009), increased the coverage of the introgressed segments of the wild accession substantially. The high-throughput genotyping occurred in advanced generations and it allowed not only an improved estimation of the extent of substitution segments and a more accurate QTL localisation using the entire population, but also the selection of a minimum number of 29 lines to represent the genome of Caesarea 26-24 (GCP project 2010, unpublished), 32 lines for the ISR42-8 genome (Schmalenbach *et al.*, 2011) and 36 lines for H602 genome (Sato and Takeda, 2009).

In regard to the RCSL population developed by Matus *et al.* (2003), the 47 SSRs were used in association analysis for domestication and malting traits scored in one growing season (Matus *et al.*, 2003) as well as for plant height, grain yield and grain yield adaptability using a multi-trial field dataset for six year/location combinations (Inostroza *et al.*, 2009). Despite the overall diminished performance associated with the wild barley introgressed regions, both studies found Caesarea 26-24 is a potential donor of favourable alleles for improving malting and agronomic traits of the recurrent parent. However, the low marker genome coverage limited the linear regression analysis to detect significant SSR–trait associations to a few loci.

An improved marker map density for 765 polymorphic SNP-markers from the Barley Oligo Pool Assay 1 (BOPA1) set was achieved as part of the Generation Challenge Programme (2010, unpublished), and a new association analysis, using a multiple QTL mapping approach and phenotypic data collected in three Chilean environments with contrasted water availability, was

conducted. As well as confirming previous associations, new insights on the favourable effect of the introgressed segments to improve grain yield under drought were found. However, these findings were not conclusive since they were based only on one season data.

This chapter will focus on the genetic dissection of quantitative traits using a subset of preselected RCSLs representing the entire genome of the wild barley accession Caesarea 26-24. Studies using minimum groups of lines defined for other RCSL populations have been effective not only to verify previously detected QTLs in the larger population (von Korff *et al.*, 2004; Wang *et al.*, 2010) but also to select introgression lines containing a target region for QTL fine mapping and map-based cloning (Schmalenbach *et al.*, 2011). With this in mind, the main objectives for the present study are: i) improve the characterisation of the extent and coverage of the wild genome in the preselected group of RCSLs using over 7000 SNP-markers from the 9K Infinium iSelect SNP platform (Comadran *et al.*, 2012); ii) establish a suitable marker-trait association analysis for a small group of RCSLs able to integrate the high-throughput genotypic information and assess effectively the genetic control of quantitative traits measured in field trials during two growing seasons (Chapter 2); iii) verify if possible the associations found in previous studies using a selected group of lines and determine the potential of Caesarea 26-24 in the improvement of the elite variety cv. Harrington for future breeding programmes.

3.2 Material and methods

3.2.1 Plant material and genotyping

The genotypic characterization of the RCSLs population (Matus *et al.*, 2003) carried out as part of the Generation Challenge Program (2010, unpublished) allowed the identification of minimum panels of introgression lines for each of the barley chromosomes using 1536 SNPs from the Barley Oligo Pool Assay 1 (BOPA1) (Close *et al.*, 2009). The group of 29 RCSLs selected for this study were representative of the entire genome of the wild barley accession (Caesarea 26-24) used as the donor parent of the population. For the present study, the set of preselected lines was characterized for a larger set of markers from 9K Infinium iSelect SNP platform (7864 gene-based SNP markers) described by Comadran *et al.*, (2012).

3.2.1.1 DNA extraction and quantification

Genomic DNA was extracted from leaf samples of ten days old seedlings (around 100 mg wet weight plant material) using the QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instructions (April 2012 version) (https://www.qiagen.com/gb/shop/sample-technologies/dna/dna-preparation/dneasy-plant-mini-kit#resources). Prior to the DNA extraction ten seeds of each genotype were germinated on filter paper moistened with 4 ml of sterile water in Petri dishes. After two days at 4^{o}C, seedlings grew for eight days at room temperature. Leaf tissue collected and bulked from 5 seedlings per genotype was frozen in liquid nitrogen and disrupted to obtain a fine powder using a micropestle.

DNA quality was checked using 1.5% agarose-gel electrophoresis prepared with 1×Tris/Borate/EDTA (TBE) electrophoresis buffer (pH 8.0) containing 10 µl SYBR®Safe DNA gel stain (Thermo Fisher Scientific, Life Technologies, USA). The gel was placed in the electrophoresis chamber (Sub-Cell®GT, Bio-Rad Laboratories, Inc.) and covered with about 5 mm 1×TBE once it was cooled and solidified. For each sample, 5 µl of DNA was loaded in the wells of the gel together with 2.5 µl 1×bromophenol blue buffer. The 1 kb λ-DNA molecular ladder (Promega, Madison, WI, USA) was used as standard for determining the DNA concentration by visual comparison. Electrophoresis was conducted at a constant voltage of 100 V for 45 minutes. The results were visualised and recorded using a UV Transilluminator.

3.2.1.2 RCSLs genotyping

Genotyping using the barley Infinium iSelect 9K SNP chip was conducted by TraitGenetics GmbH (Gatersleben, Germany) using DNA samples at a concentration of 50 ng/µl. This genotyping array comprises 2832 barley oligo pool assay (BOPA1 and BOPA2) SNPs, 5010 SNPs developed from next generation sequencing data and 22 SNPs from resequencing studies (Comadran *et al.*, 2012). The assay is based on the hybridisation of whole-genome-amplified (WGA) genomic DNA to a bead array of locus-specific primers. The locus-specific hybridisation is determined by an allele-specific single-base extension assay and nucleotide-specific fluorescence staining in which signal is amplified, analysed and translated to generate a raw data file containing the hybridisation intensity readings or genotype calls.

The Illumina GenomeStudio Genotyping (GT) module (Illumina, San Diego, CA) (http://support.illumina.com/array/array_software/genomestudio.html) and Flapjack (Milne *et al.*, 2010) were used for the analysis of the genotype calls generated. Briefly, the data obtained from the Infinium assay was visualised using the GenomeStudio Genoplot where the genotype calls were automatically plotted depending on the hybridisation signal intensity and the allele frequency. This module integrates a series of algorithms that allow the normalisation of the raw data obtained from the genotyping array analysis as well as for clustering the genotypes in three possible groups: heterozygous (AB) and homozygous genotypes (AA & BB). Each genotype call is scored according the results of this analysis (the genotype calls algorithms and the fitting of the data) in a GenCall score which gives an indication of the confidence with which the genotypes calls have occurred allowing the identification of failed (score<0.2) and possibly ambiguous (score<0.7) results. These were revised or removed for the final selection of markers. Also, heterozygous genotype calls were strictly revised since we expect homozygosity in the RCSLs. The set of polymorphic SNP markers was defined after discarding the markers that were monomorphic as well as those unmapped and markers with more than 10% missing data in this group of lines.

The molecular marker data was then imported to Graphical Genotypes software, GGT 2.0 (van Berloo, 2008), in order to visualise and characterise the proportion of exotic chromosomal regions from the donor parent introgressed into the elite barley genetic background for each of the lines. In this case, SNP allelic information was coded as '*A*' for the recurrent parent allelic variant in a locus, '*B*' for the donor parent alleles and '*?*' for the missing data. Additionally, PAST version 2.17c (Hammer *et al.*, 2001) was used to perform a cluster analysis using the unweighted pair group average (UPGMA) to define the genotypic distance among the RCSLs and both parents based on the Hamming's distance (simple matching). This approach computes

the distance between genotypes based on a simple mismatching analysis between binary sequences of data, that is by counting the number of loci where individuals have different SNP allelic variants. In this case 1s and 0s were used for coding the recurrent and the donor parent alleles respectively.

3.2.2 RCSLs phenotypic characterisation

The twenty-nine selected RCSLs were phenotyped in two field trials (2013 and 2014) for three water regimes. Experiments were carried out using a rain-out shelter to protect the plots subjected to water deficit from rainfall and to have better control over the irrigated treatments. The experiment was established in a row column design with the treatment and replicates superimposed. The water regimes applied aimed to evaluate the impact of water deficit on the RCSLs performance. Chapter 2 describes in detail the layout of the experiment and the phenotypic characterization of the lines throughout the two growing seasons. The marker–trait association analysis was performed for thirteen traits (Table 3.1) explained in detail in Table 2.2.

Table 3.1 Traits used in the marker–trait association analysis

Morphological traits	***Code***
Collar Height (cm)	COL
Peduncle length (cm)	PdL
Peduncle extrusion (cm)	PdE
Ear Length (cm)	EAR
Seed Width (mm)	SdW
Seed Length (mm)	SdL
Seed Area (mm^2)	SdA
Developmental traits	
Heading date (DAS)	HEA
Number of tillers	TILL
Agronomic traits	
Dry yield ($kg.ha^{-1}$)	DY
Biomass yield ($kg.ha^{-1}$)	BY
Thousand grain weight (g)	TGW
Harvest index (%)	HI

3.2.3 QTL analysis

3.2.3.1 Statistical model and marker selection

The association analysis between SNP markers and phenotypic traits was carried out with GenStat 17th Edition (VSN International, UK) by integrating the molecular marker information into a mixed model to test the effect of DNA polymorphism on the phenotypic trait variation. The estimates for fixed and random parameters of the mixed model were obtained by residual maximum likelihood (REML) method (Payne *et al.*, 2011) as described in Chapter 2. Because the interest was in genetic variation across the RCSLs rather than the genotypes themselves, the lines were assumed as random effects, whereas the SNP marker data was included as part of the fixed effect along with year, water treatment and their interaction. The analysis was conducted for one marker at a time in a loop computed for each trait. The statistical significance for the fixed model effects was assessed using a chi-square based Wald-test (Appendix 7). The trait response ($Y_{ijklmno}$) was calculated according to the following hierarchical model:

Equation 4

$$Y_{ijklmno} = \mu + M_i + T_j + M_i * T_j + Y_k + M_i * Y_k + T_j * Y_k + R_l(T_j * Y_k) + C_m(T_j * Y_k * R_l) + G_n + \varepsilon_{o(ijklmn)}$$

where μ is the general mean, M_i is the fixed effect of the *i*-th SNP marker, T_j is the fixed effect of the *j*-th water treatment, M_i*T_j is the fixed effect of the interaction of the *i*-th SNP marker and the *j*-th treatment, Y_k is the fixed effect of the *k*-th year, M_i*Y_k is the fixed effect of the interaction of the *i*-th genotype and the *k*-th year, T_j*Y_k is the fixed effect of the interaction of the *j*-th treatment and the *k*-th year, R_l(T_j*Y_k) is the random effect of the *l*-th replicate nested in *j*-th treatment and *k*-th year, C_m(T_j*Y_k*R_l) is the random effect of the *m*-th column nested in the *j*-th treatment, *k*-th year and *l*-th replicate, G_n is the random effect of the *n*-th genotype and $\varepsilon_{o(ijklmn)}$ is the residual term of $X_{ijklmno}$.

This QTL mapping strategy can be defined as a REML single-locus analysis similar to the one described in Bauer *et al.* (2009). The approach follows the principles of regression-based QTL mapping analysis that are normally used for mapping populations derived from bi-parental crosses such as single interval mapping (SIM) (Lander and Botstein, 1989) or composite interval mapping (CIM) (Jansen *et al.*, 1995). However, due to the small size of the group of RCSLs selected for the present study, conventional QTL mapping methods cannot be adopted and a hierarchical mixed model was considered a more suitable method for performing a marker–trait association analysis. The REML analysis allows major QTLs or chromosome regions with stable effect across environments (marker main effect level), minor QTLs which

effects are modulated in response to the environment (marker treatment interaction effect level) to be defined and also infers genetic correlation between traits derived from QTLs linked to several of these (pleiotropic effects).

The marker–trait association analysis was performed using a subset of marker data which was also recoded in two classes: donor parent alleles as 0s and recurrent parent alleles as 1s. In order to simplify the computational process, blocks of contiguous markers that were polymorphic for the same RCSLs were treated as single entity (i.e. one SNP representing a block of SNPs). This way, redundant markers were removed from the analysis, but their map information was considered to define the size of the chromosome regions associated with the phenotype observed. Since the wild barley introgressions overlapped across contiguous regions within a chromosome, the length of each genomic region tested was determined as the genetic distance between the first SNP markers defining adjacent loci.

3.2.3.2 QTL location

Markers with significant main effects and/or interactions with the treatment at 0.05(*), 0.01(**) and 0.001(***) levels of significance were considered for QTL location. Because an aim of the study was to identify stable marker–trait associations across experiments and treatments, the SNP × Year interactions were not investigated further.

The *p* value of the most strongly associated SNP marker–trait within a chromosome region was used to define the peak region of the QTL. The Best Linear Unbiased Estimates (BLUEs) for each trait for the donor ([*Hsp*]) and the recurrent parent ([*Hv*]) alleles obtained for the significant peak region were used to calculate the relative contribution (RP) of the exotic parent alleles on the trait performance as follows:

Equation 5

$$RP(\%) = \frac{[Hsp] - [Hv]}{[Hv]} * 100$$

Additionally, the proportion of the phenotypic variance explained by the marker and the marker by treatment interaction (R^2) was computed. Neighbouring blocks of markers showing significant effects in the same direction were assumed to be part of the same QTL and used to define the significant region of the marker–trait association.

The position of major determinant genes related to plant phenology and morphology was estimated from previous studies and public gene databases to support the result obtain from this analysis. Additionally, some genomic regions accounting for a significantly high proportion of a trait's variance were used to identify possible putative candidate genes underlying the predicted phenotypic variation. Firstly, the number of genes in the target regions was estimated by looking at the physical position of the markers in the barley new assembly genome (IBSC, 2016). Secondly, the putative genes content was compiled by looking at the annotated high confidence genes between the two flanking markers defined for the region of interest. Finally, possible candidates were identified by examining gene ontology annotations (GO) and the homologies found in other crop relative species genomes using Basic Local Alignment Search Tool (BLAST) analysis on the NCBI website: https://www.ncbi.nlm.nih.gov/. In this case, the BARLEYMAP pipeline (Cantalapiedra *et al.*, 2015), based on the previous IBSC (2012) map assembly and the POPSEQ map of Morex contigs (Mascher *et al.*, 2013a), was used as a reference to identify the barley predicted transcripts (MLOCs) which could be potential candidates according to their gene function descriptors and GO annotations for the various QTLs.

3.3 Results

3.3.1 RCSLs genotypic characterisation

The RCSLs were successfully genotyped using the 9K Infinium iSelect SNP chip (Comadran *et al.*, 2012). Markers with unmapped position (42.9%), monomorphic between the two parents or across the RCSLs (29.1%) and with more than 10% missing data (1%) were removed from the analysis. Also, a small group of seven polymorphic markers on 1H and 5H were removed due to ambiguous results (Appendix 8). The resulting genetic map consisted of 1848 SNP markers with a total length of 988.8 cM (Appendix 9, CD-ROM). Genetic position of the markers was determined using the Morex × Barke RILs population (Comadran *et al.*, 2012). Only one marker, (SCRI_RS_163112) was re-positioned to 4H at 0.8 cM from 2H at 149.2 cM following correct BLAST analysis. The mean distance between pairs of markers was 0.56 cM, showing a maximum genetic distance of 14.9 cM on top of chromosome 4H, although since markers where inherited as a block this estimation might be misled. Chromosome 4H accounted for the fewest polymorphic SNP markers (151). In contrast, chromosome 5H was characterised for the largest group of SNPs (335).

Table 3.2. Summary of polymorphic SNP markers defining the RCSL genetic map

Chr.	No. markers	Length (cM)	Markers Av. distance (cM)[1]	Markers Max. distance (cM)[2]	RCSLs with unique *Hsp* regions
1H	186	133.0	0.72	7.60	OSU015
2H	328	149.4	0.46	8.60	-
3H	321	154.9	0.48	9.60	OSU048 / OSU033
4H	158	115.2	0.73	14.90	OSU038 / OSU086
5H	335	168.8	0.50	9.40	OSU035 / OSU065
6H	281	126.6	0.45	10.20	OSU065
7H	239	140.9	0.59	6.40	-
Total	*1848*	*988.8*	*0.54*	*14.90*	

[1]Average distance between markers per chromosome
[2] Maximum distance between markers within chromosome

As a result, the chromosome introgressions from the wild barley genome almost covered the entire genome, with the exception of a small region on 5H between 168.9 and 169.4 cM. The RCSLs generally harbour one exotic introgression on more than one chromosome (6.4±0.4 average number of *Hsp* introgressions per line) and in some cases, up to three introgressions per chromosome (Appendix 10). On average, the percentage of wild barley genome introgressed in the RCSLs corresponded to 13.4%, but ranged from a few centimorgans to almost half a

chromosome. For example, OSU012 has 48.3% of wild barley on chromosome 2H and OSU065 has 65.8% on chromosome 6H. In contrast, OSU053 had the smallest donor parent regions, with only 3.7% of wild barley genome represented. Overall OSU033 and OSU065 had the largest contribution from the wild barley genome (26.6% and 23.9% respectively) with *Hsp* regions on seven chromosomes spanning a total length of 263.2 cM in the case of OSU033 and 236.4 cM in OSU065 with introgressions in six out of seven chromosomes, whereas OSU053 had five introgressions covering only 36.6 cM on four chromosomes (1H, 2H, 6H and 7H) (Fig. 3.2).

The dendrogram obtained from the cluster analysis illustrates the genetic relatedness among the RCSLs and parents (Fig. 3.3). In effect, genotypes OSU033 and OSU065 appear to be genetically distant from cv. Harrington (as expected from the large wild barley introgressions) and the remaining RCSLs group together gradually differing from the recurrent parent with OSU060 being most similar to the elite parent.

Two RCSLs (OSU015 and OSU016) were found to be genetically identical, probably due to an error in labelling the samples. The comparison with the results obtained in previous studies with a smaller set of SNP markers (BOPA1) aided in determining which line was correct. Consequently, OSU016 was removed and the QTL analysis was computed using genotypic and phenotypic data of 28 RCSLs and cv. Harrington. Fortunately, removing OSU016 from the set of 29 RCSLs did not leave any gap in the representation of the wild barley genome. The allele frequency of the minor allele for this set of lines ranged from 0.036 or 3.6% to 0.286 or 28.6% (Appendix 11). Eight unique chromosome regions represented exclusively by one RCSL were found in five out of seven chromosomes (Table 3.2) whereas up to eight RCSLs shared *Hsp* alleles in four regions of the genome.

Set of markers selected for QTL analysis

The association analysis was performed for 235 defined chromosome regions (Fig. 3.4). Each of these loci was represented by a group or block of SNP markers for which the RCSLs showed the same genotype (4.1±0.1 RCSLs on average with exotic alleles per chromosome region tested). The number of genomic regions assessed per chromosome ranged from 28 (4H) to 39 (2H) with a mean length of 4.2±0.3 cM and a maximum of 20.4 cM on chromosome 2H (Fig. 3.5 and Appendix 12).

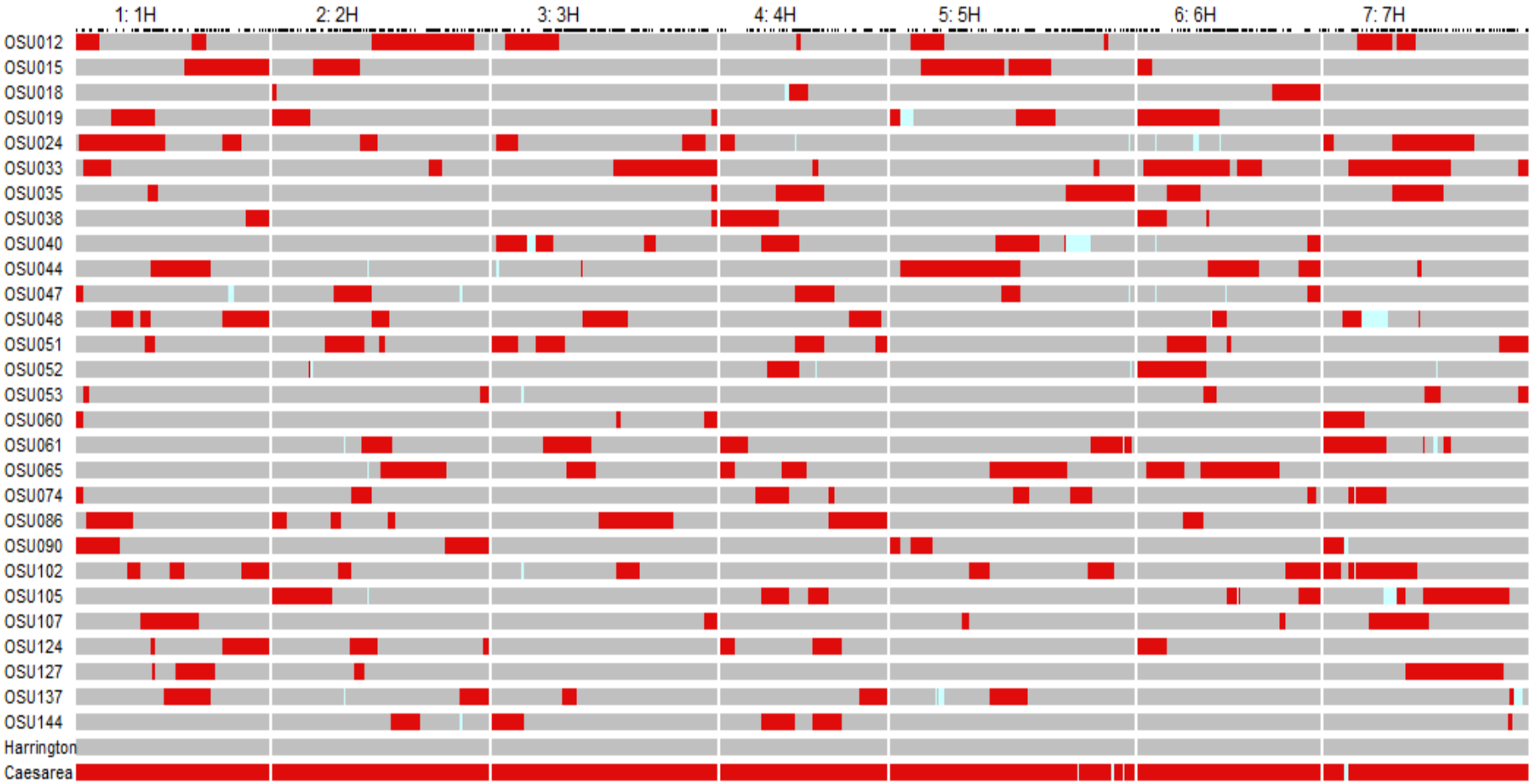

Figure 3.1 . Graphical genotypes of Caesarea 26-24, cv. Harrington and the 28 RCSLs used in the association analysis study.
Caesarea 26-24 genome and substituted segments in the RCSLs are represented in red; cv. Harrington genome and genetic background in the RCSLs in grey. Missing marker data are indicated in light blue. Each chromosome is oriented with the left arms from the left.

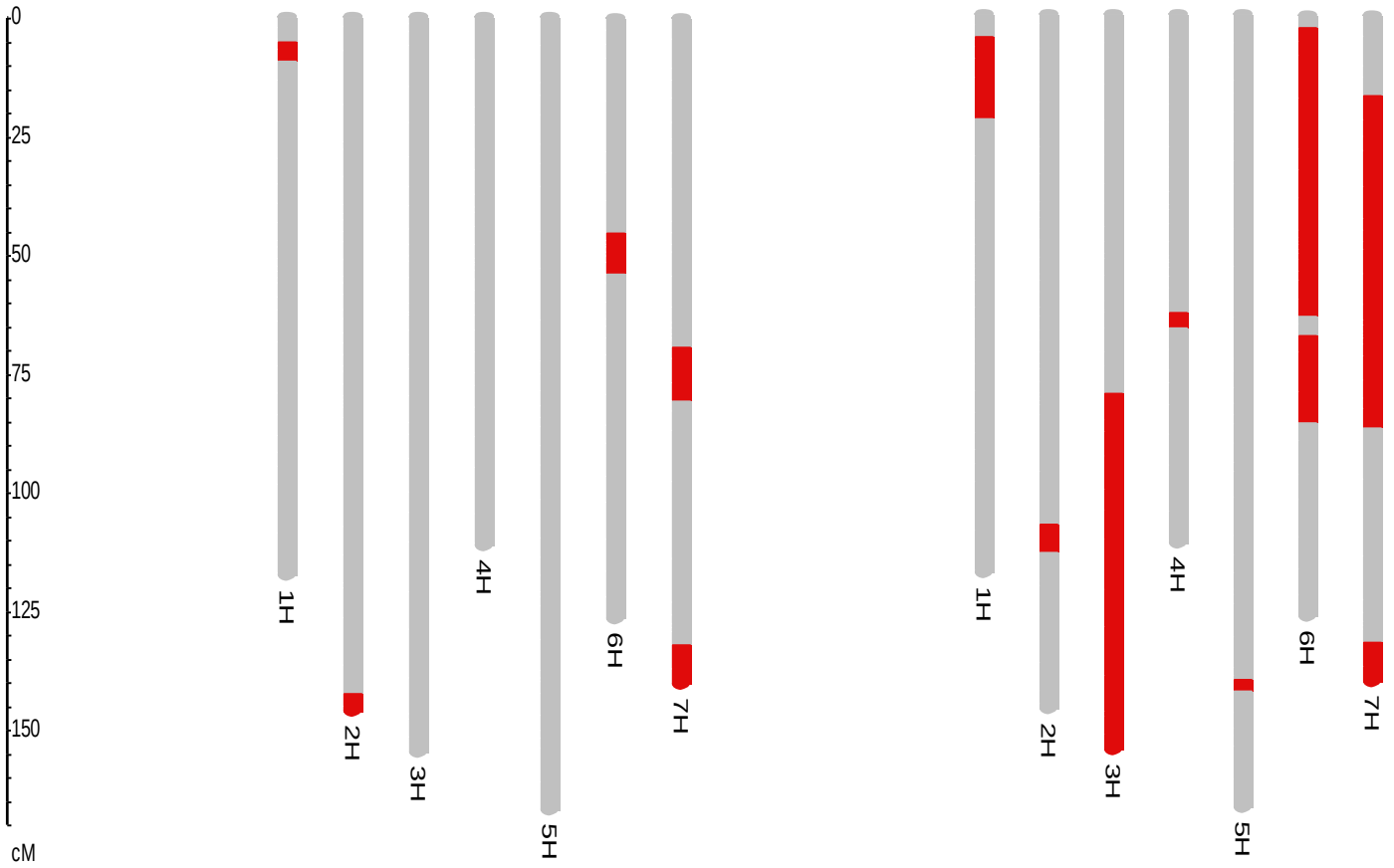

Figure 3.2 Graphical genotypes for the two extreme RCSLs.
The exotic alleles (in red) represent 3.7% and 26.6% of OSU053 (left) and OSU033 (right) genomes respectively

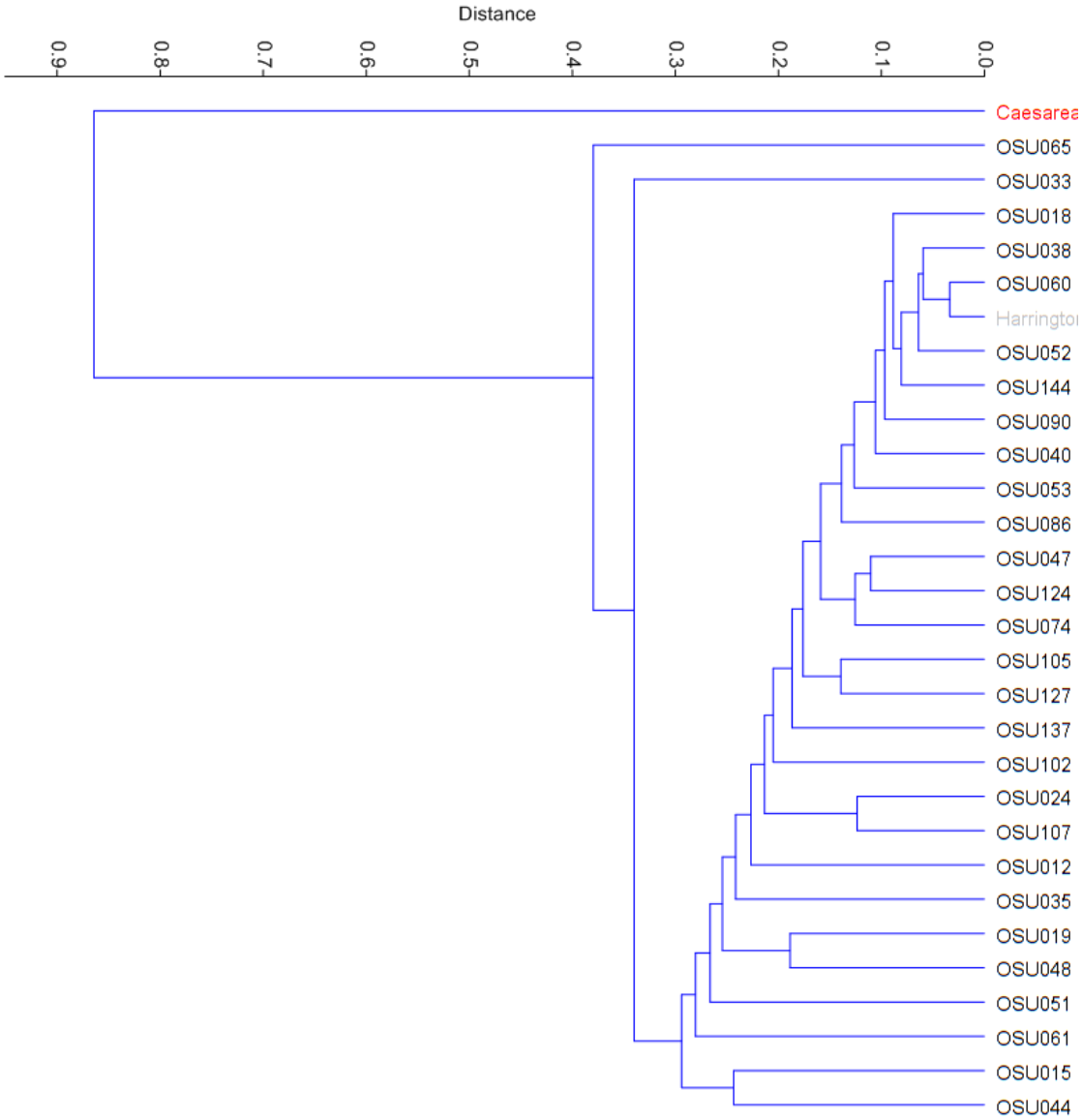

Figure 3.3 UPGMA clustering of 28 RCSLs, cv. Harrington (recurrent parent) and Caesarea 26-24 (donor parent) by similarity coefficients of allele sharing based on Hamming's distance for 1848 SNP markers.

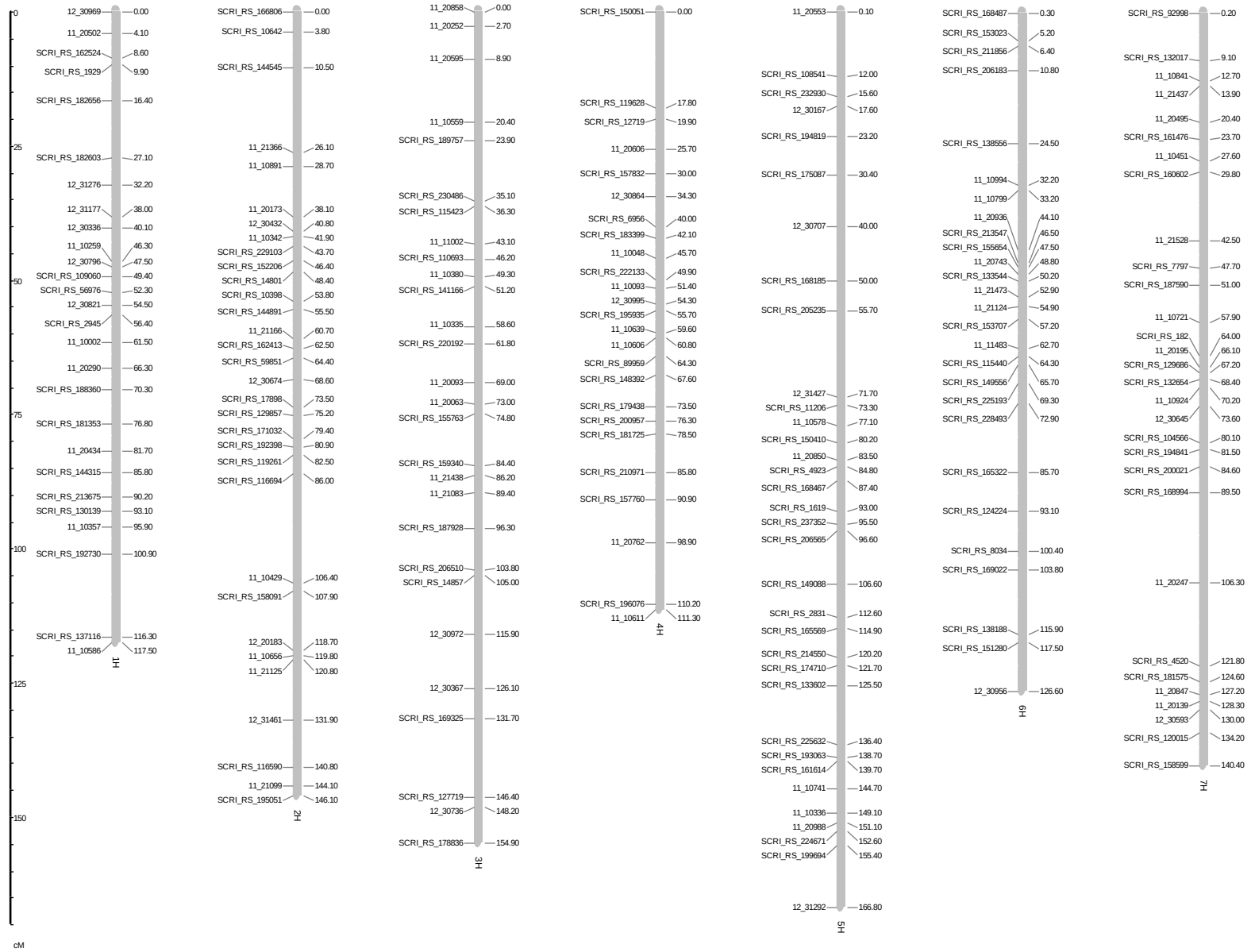

Figure 3.4. iSelect SNP markers representing the 235 block of markers used in the REML single locus association analysis.
Markers names are given on the left side of the chromosome bar and the genetic position (cM) on the right based on Comadran *et al.* (2012).

3.3.2 QTL analysis results

The result obtained from the association analysis performed for thirteen traits measured across two growing seasons revealed a total of 161 significant (P<0.05) marker–trait associations from 3055 possible combinations (Appendix 13, CD-ROM). Tables 3.4, 3.5 and 3.6 show a summary of the QTLs identified for morphological, developmental and agronomic traits respectively. Eighty one loci were significantly associated with barley morphological traits (COL, PdL, PdE, SdW, SdL, SdA, EAR), 22 with developmental characteristics (HEA, TILL) and 58 with the agronomic performance of the crop (DY, TGW, BY, HI). Marker main effect accounted for 47.2% of loci–trait associations, with 45.3% defined by the interaction with treatment. In some cases, both effect levels were determined for the same genomic region (7.5%). The size of the main genomic region associated with a trait (QTL peak region) ranged from 0 cM (in TGW6) to 17.8 cM (in TGW9) with an average of 5.9 cM. The chromosome regions identified as significantly affecting the phenotype ranged from 0.1 cM to 62 cM (in TILL3 and HI9 respectively), with an average of 14.5 cM.

Overall the exotic wild barley alleles were found to increase the trait performance in 55.3% of the loci associations compared with the 44.7% where the exotic genome was found to reduce the phenotype (Table 3.3).

Table 3.3. Number of QTLs detected for each trait
QTLs are grouped following two criteria: the association effect defined for the marker and the relative performance of the exotic alleles compared to the elite parent alleles.

		Association effect[1]			*Hsp* RP [2]		*Total (%)*
Trait	Code	M	MxT	both	↑	↓	
Collar height	COL	8	11	1	13	7	*20 (12.4%)*
Peduncle Length	PdL	5	7	0	11	1	*12 (7.5%)*
Peduncle extrusion	PdE	4	5	1	10	0	*10 (6.2%)*
Seed Width	SdW	8	4	0	7	5	*12 (7.5%)*
Seed Length	SdL	2	5	2	6	3	*9 (5.6%)*
Seed Area	SdA	2	4	2	7	1	*8 (5.0%)*
Ear length	EAR	5	4	1	7	3	*10 (6.2%)*
Heading date	HEA	5	6	1	5	7	*12 (7.5%)*
Number of tillers	TILL	6	4	0	2	8	*10 (6.2%)*
Dry yield	DY	10	5	0	2	13	*15 (9.3%)*
Thousand Grain Weight	TGW	8	7	1	14	2	*16 (9.9%)*
Biomass yield	BY	5	3	1	4	5	*9 (5.6%)*
Harvest index	HI	8	8	2	1	17	*18 (11.2%)*
	Total	*76*	*73*	*12*	*89*	*72*	*161*
	(%)	*(47.2%)*	*(45.3%)*	*(7.5%)*	*(55.3%)*	*(44.7%)*	*(100%)*

1. Number of marker-trait associations identified for the marker main effect (M), the marker–treatment interaction (MxT) or both
2. Effect of the wild barley alleles (*Hsp*) on the relative performance estimated for a trait: increased estimated mean (↑) or decreased estimated mean (↓) compared to the effect of the elite cultivar (*Hv*) performance

3.3.2.1 Morphological traits

i. Collar Height (COL)

Twenty significant marker trait associations for collar height (COL) were identified in 6 of the chromosomes (none on 4H). Eight of these associations were defined as marker main effect, eleven for interaction with the treatment and one at both levels of effect, M and MxT (Table 3.4). The QTLs COL10 (3H), COL14 (5H) and COL16 (6H) were identified as the most significant ($P<0.001$) associations for the marker main effect. Each of these three loci explained a large proportion of the phenotypic variance (30.5%, 31.25% and 38.1% respectively). The marker–treatment interaction effect on collar height was generally less significant; however COL1 and COL5 explained around 1% of the phenotypic variation (Table 3.4).

The wild barley alleles broadly contributed to increase collar height in the loci identified (14 QTLs). For example, exotic alleles at COL10 (3H) increased collar height by 26.7% (27.1 cm) in relation to the elite barley alleles. In contrast the exotic alleles were also found to reduce collar height at two loci main effect, COL4 (2H) and COL20 (7H), and at four loci in the interaction with the treatment, COL2 (1H), COL3 (2H), COL15 (6H) and COL19 (7H). The exotic alleles on COL19 were associated with the greatest reduction in collar height compared to the recurrent parent alleles (10.4% reduction, 10.7 cm).

ii. Peduncle length and extrusion (PdL and PdE)

Ten marker–trait associations were significant for peduncle extrusion (PdE) on chromosomes 2H, 3H, 4H, 5H and 7H (the majority of them in common with the associations defined for peduncle length) (Table 3.4). The most significant association (PdE3, $P<0.001$) was identified on chromosome 3H for the marker main effect which explained 32.3% of the phenotypic variation. In this case the exotic alleles doubled the length of the peduncle in comparison with the elite alleles at the locus (50.1% increase, 6.7 cm longer). Overall, the presence of exotic alleles increased the length of the peduncle at the loci identified (9 of the 10 QTL).

iii. Seed width (SdW)

The phenotypic variation found for seed width was associated with twelve genomic regions on all chromosomes except 7H, predominantly for the marker main effect (8 QTL). SdW1 (1H) showed the most significant association ($P<0.001$) for the trait, explaining 35.6% of the variation. In this case the exotic alleles contributed to a decrease in seed width 0.12 mm (3.3%). However, in most of the other QTLs the exotic alleles were associated with a significant increased width. The largest effect was found on 5H (SdW11) with an increase of up to 4.3% of its width ($P<0.01$).

iv. Seed Length (SdL)

Nine loci were found significantly associated with the Seed length (SdL). The strongest associations (P<0.001) were found for the marker main effect at SdL1 (1H) and SdL7 (5H). The peak marker at these loci explained 81% and 53.4% of the trait phenotypic variation respectively. In both cases the exotic alleles increased the length of the seed by 2.7 mm (32.7%) at SdL1 (1H) and 1.6 mm (53.4%) at SdL7 (5H). The effect in the interaction with the treatment at these two regions was also significant. Similarly, SdL3 (2H) and SdL8 (5H) accounted for large proportion of the phenotypic variation (44% and 34.6% respectively) with the exotic alleles contributing to increase the length of the seed.

Table 3.4 List of QTLs identified for the morphological traits collar height (COL), peduncle extrusion (PdE), seed width (SdW) and seed length (SdL)

Trait	QTL	Peak marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP[7] (%)	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						SNP	SNPxT		[*Hsp*]	[*Hv*]				
COL	COL1	SCRI_RS_1929	1H	9.9–16.4	MxT	ns	***	D	98.16	94.01	4.42	1.04*	5.0–27.1	22.10
								FI	113.03	104.24	8.43			
								PI	112.77	104.65	7.75			
	COL2	SCRI_RS_207335	1H	52.7–54.5	MxT	ns	*	D	93.86	95.05	-1.25	0.37*	52.7–54.5	1.80
								FI	102.79	106.89	-3.83			
								PI	103.72	106.94	-3.00			
	COL3	SCRI_RS_144545	2H	10.5–26.1	MxT	ns	**	D	90.10	95.06	-5.22	0.96*	10.5–26.2	15.70
								FI	94.80	106.56	-11.04			
								PI	96.17	106.79	-9.94			
	COL4	SCRI_RS_170235	2H	56.2–60.7	M	*	ns	n/a	95.30	103.97	-8.34	13.64	56.2–60.7	4.50
	COL5	11_10475	2H	81.5–82.5	MxT	ns	***	D	97.47	94.15	3.53	1.42*	72.2–120.8	48.60
								FI	112.18	104.42	7.44			
								PI	113.04	104.60	8.07			
	COL6	SCRI_RS_158091	2H	107.9–118.7	M	**	**	n/a	116.42	100.53	15.81	29.22	107.9–118.7	10.80
	COL7	11_20595	3H	8.9–20.4	MxT	ns	*	D	95.12	94.64	0.50	0.59*	8.9–20.4	11.50
								FI	109.19	105.04	3.95			
								PI	109.17	105.41	3.57			
	COL8	11_11002	3H	43.1–46.2	MxT	ns	*	D	92.29	95.00	-2.85	0.39*	43.1–46.2	3.10
								FI	106.30	105.69	0.58			
								PI	107.36	105.90	1.37			
	COL9	11_10335	3H	58.6–61.8	M	*	ns	n/a	114.34	101.27	12.90	11.77	58.6–62.7	4.10
	COL10	12_30367	3H	126.1–131.7	M	***	ns	n/a	128.30	101.20	26.70	30.46	103.8–148.2	44.40
	COL11	SCRI_RS_169325	3H	131.7–146.4	MxT	**	*	D	109.52	93.63	16.98	0.56*	131.7–147.2	15.50
								FI	125.94	104.26	20.79			
								PI	124.03	104.72	18.44			
	COL12	SCRI_RS_108541	5H	12.0–15.6	MxT	ns	*	D	100.67	94.75	6.25	1.02*	12.0–15.6	3.60
								FI	105.25	106.29	-0.97			
								PI	107.63	106.50	1.06			
	COL13	SCRI_RS_214550	5H	120.2–121.7	M	*	ns	n/a	114.96	101.23	13.57	13.31	120.2–122.4	2.20
	COL14	SCRI_RS_44795	5H	140.1–144.7	M	***	ns	n/a	114.66	100.18	14.46	31.25	140.1–144.7	4.60

Table 3.4 Continued

Trait	QTL	Peak marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP[7] (%)	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						SNP	SNPxT		[*Hsp*]	[*Hv*]				
	COL15	SCRI_RS_168487	6H	0.3–5.2	MxT	ns	*	D	92.34	95.22	-3.02	0.48*	0.3–5.2	4.90
								FI	101.23	106.70	-5.12			
								PI	100.62	107.19	-6.13			
	COL16	SCRI_RS_165322	6H	85.7–86.3	M	***	*	n/a	122.23	100.69	21.39	38.10	57.2–86.3	29.10
		SCRI_RS_165322	6H	85.7–86.3	MxT	***	*	D	112.09	93.43	19.97	0.42*	85.7–86.3	0.60
								FI	127.84	104.12	22.78			
								PI	126.76	104.52	21.28			
	COL17	SCRI_RS_160602	7H	29.8–42.5	MxT	*	*	D	100.78	93.52	7.76	0.72*	23.7–48.0	24.30
								FI	114.92	103.86	10.64			
								PI	114.63	104.46	9.73			
	COL18	SCRI_RS_150016	7H	48.0–51.0	M	**	ns	n/a	112.28	100.22	12.04	24.03	29.8–70.2	40.40
	COL19	11_20247	7H	106.3–121.8	MxT	ns	*	D	89.96	95.08	-5.38	0.48*	106.3–121.8	15.50
								FI	95.79	106.49	-10.05			
								PI	97.92	106.65	-8.19			
	COL20	SCRI_RS_4520	7H	121.8–124.6	M	*	ns	n/a	92.56	103.29	-10.39	11.28	121.8–124.6	2.80
PdE	PdE1	SCRI_RS_192398	2H	80.9–81.5	MxT	ns	**	D	12.15	11.06	9.91	1.76*	73.7–86.0	12.30
								FI	17.67	14.55	21.43			
								PI	17.91	14.72	21.73			
	PdE2	SCRI_RS_115423	3H	36.3–43.1	M	*	**	n/a	16.67	13.32	25.12	12.94	36.3–43.1	6.80
		SCRI_RS_115423	3H	36.3–43.1	MxT	*	**	D	12.78	10.96	16.64	2.12*	8.9–46.2	37.30
								FI	18.51	14.42	28.41			
								PI	18.71	14.59	28.23			
	PdE3	SCRI_RS_141166	3H	51.2–51.3	M	***	ns	n/a	20.00	13.32	50.14	32.28	49.3–62.7	13.40
	PdE4	11_10611	4H	111.3–115.2	MxT	ns	*	D	11.10	11.22	-1.09	0.98*	111.3–115.2	3.90
								FI	15.99	14.86	7.56			
								PI	16.97	14.95	13.55			
	PdE5	11_10336	5H	149.1–151.1	MxT	ns	*	D	11.10	11.22	17.77	0.88*	149.1–151.1	2.00
								FI	15.99	14.86	26.89			
								PI	16.97	14.95	19.60			
	PdE6	SCRI_RS_199694	5H	155.4–166.8	M	**	ns	n/a	18.99	13.40	41.78	21.65	139.7–166.8	27.10
	PdE7	SCRI_RS_160602	7H	29.8–42.5	M	*	**	n/a	16.23	13.40	21.12	12.00	29.8–42.5	12.70

Table 3.4 Continued

Trait	QTL	Peak marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP[7] (%)	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						SNP	SNPxT		[*Hsp*]	[*Hv*]				
PdE (cont)	PdE8	11_21528	7H	42.5–43.3	MxT	ns	**	D	12.09	10.85	11.42	13.48	20.4–48.0	27.60
								FI	17.67	14.20	24.48			
								PI	16.94	14.67	15.46			
	PdE9	SCRI_RS_200021	7H	84.6–89.5	M	**	ns	n/a	17.39	13.03	33.46	30.19	68.4–89.5	21.10
	PdE10	SCRI_RS_4520	7H	121.8–124.6	MxT	ns	*	D	12.39	11.07	11.88	1.15*	106.3–124.6	18.30
								FI	15.40	14.93	3.13			
								PI	17.61	14.88	18.37			
SdW	SdW1	SCRI_RS_181353	1H	76.8–81.7	M	***	ns	n/a	3.45	3.56	-3.31	35.57	52.3–93.1	40.80
	SdW2	SCRI_RS_170235	2H	56.2–60.7	M	*	ns	n/a	3.49	3.56	-2.03	13.30	53.8–60.7	6.90
	SdW3	SCRI_RS_119261	2H	82.5–86.0	M	*	ns	n/a	3.61	3.53	2.15	10.02	82.5–86.0	3.50
	SdW4	11_21099	2H	144.1–146.1	MxT	ns	**	D	3.59	3.56	0.95	2.23*	140.8–146.1	5.30
								FI	3.51	3.54	-0.82			
								PI	3.50	3.54	-0.98			
	SdW5	SCRI_RS_189757	3H	23.9–35.1	M	**	ns	n/a	3.67	3.53	3.87	19.91	20.4–35.1	14.70
	SdW6	SCRI_RS_14857	3H	105.0–115.9	M	*	ns	n/a	3.63	3.53	2.59	11.80	105.0–115.9	10.90
	SdW7	SCRI_RS_222133	4H	49.9–51.4	M	**	ns	n/a	3.62	3.52	2.85	29.05	34.3–54.3	20.00
	SdW8	12_30995	4H	54.3–55.7	MxT	ns	**	D	3.55	3.57	-0.37	2.20*	54.3–60.8	6.50
								FI	3.56	3.53	1.10			
								PI	3.56	3.53	0.98			
	SdW9	11_20553	5H	0.1–12.0	MxT	ns	*	D	3.61	3.56	1.34	1.65*		
								FI	3.52	3.53	-0.46			
								PI	3.50	3.53	-0.86			
	SdW10	SCRI_RS_168185	5H	50.0-55.7	M	**	ns	n/a	3.43	3.56	-3.49	24.67	40.0–73.3	33.30
	SdW11	SCRI_RS_214550	5H	120.2-121.7	M	**	ns	n/a	3.68	3.53	4.28	25.48	120.2–121.7	1.50
	SdW12	SCRI_RS_124224	6H	93.1–100.4	MxT	ns	*	D	3.61	3.56	1.26	1.44*	93.1–103.8	10.70
								FI	3.61	3.53	2.43			
								PI	3.65	3.52	3.57			
SdL	SdL1	11_10357	1H	95.9–100.9	M	***	***	n/a	11.08	8.35	32.67	80.98	76.8–133.0	56.20
		11_10357	1H	95.9-100.9	MxT	***	***	D	10.69	8.37	27.65	1.09*	90.2–133.0	42.80
								FI	11.11	8.21	35.38			
								PI	11.43	8.46	35.00			

Table 3.4 Continued

Trait	QTL	Peak marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP (%)[7]	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						M	MxT		[*Hsp*]	[*Hv*]				
SdL (cont)	SdL2	SCRI_RS_144545	2H	10.5–26.1	MxT	ns	*	D	8.73	8.43	3.55	0.55*	3.8–26.1	22.30
								FI	8.26	8.31	-0.60			
								PI	8.59	8.56	0.28			
	SdL3	11_10891	2H	28.7–38.1	M	***	ns	n/a	9.81	8.34	17.68	44.03	28.7–60.7	32.00
	SdL4	11_10342	2H	41.9–43.7	MxT	**	**	D	9.19	8.37	9.85	1.00*	40.8–48.4	7.60
								FI	9.21	8.20	12.23			
								PI	9.65	8.44	14.39			
	SdL5	SCRI_RS_14857	3H	105.0–115.9	MxT	ns	*	D	8.43	8.45	-0.25	0.54*	96.3–115.9	19.60
								FI	8.13	8.33	-2.35			
								PI	8.66	8.55	1.27			
	SdL6	SCRI_RS_210971	4H	85.8–90.9	MxT	ns	*	D	8.16	8.46	-3.55	0.60*	85.8–90.9	5.10
								FI	7.94	8.32	-4.62			
								PI	8.67	8.56	1.33			
	SdL7	12_30707	5H	40.0–50.0	M	***	**	n/a	9.94	8.33	19.36	53.44	23.2–80.2	57.00
		12_30707	5H	40.0–50.0	MxT	***	**	D	9.71	8.36	16.11	0.95*	40.0–84.8	44.80
								FI	9.98	8.18	21.95			
								PI	10.14	8.45	20.07			
	SdL8	SCRI_RS_149088	5H	106.6–112.6	M	***	ns	n/a	9.43	8.33	13.19	34.64	83.5–112.6	29.10
	SdL9	11_21528	7H	42.5–43.3	MxT	ns	*	D	8.45	8.44	0.09	0.71*	42.5–43.3	0.80
								FI	8.15	8.35	-2.36			
								PI	8.44	8.60	-1.80			

[1] Marker representing the block of markers defining the QTL peak
[2] Main effect region defined by the block of markers for the QTL peak
[3] Variation of the trait explained by the marker (M) or the marker–treatment interaction (MxT)
[4] Level of significance of the marker main effect (M) and the marker–treatment interaction (MxT). ***P<0.001,**P<0.01,*P<0.05, ns: not significant
[5] Treatment considered for the estimated means: drought (D), full irrigation (FI), partial irrigation (PI). Non applicable when means were estimated at the marker main effect level
[6] Estimated means (BLUEs) for each trait for the the donor ([*Hsp*]) and the recurrent parent ([*Hv*]) alleles at the peak QTL region
[7] Relative performance (RP) of the exotic parent alleles on the trait
[8] Variation of the trait explained by the marker and the marker–treatment interaction (*).Note that due to the linkage disequilibrium among the markers, R^2 values cannot be added together to give an estimate of the overall (%) variance explained.
[9] QTL significance region considering the blocks of markers flanking the QTL peak block
[10] Size of the QTL significance region

3.3.2.2 Developmental traits

i. Heading date (HEA)

Twelve loci were significantly associated with heading date (HEA) on all chromosomes except 6H. Five associations for marker main effect and six for the interaction with the treatment were observed (Table 3.5). One of the QTLs showed both effects (HEA2 on 2H) accounting for the maximum phenotypic variance associated with the trait for the marker main effect (51.1%). Also, a chromosomal region on 7H (HEA11) was found to explain a large proportion of the variation in the phenotype (46.8%). Therefore, the peak markers defined for HEA2 and HEA11 were identified as the most significantly associated ($P<0.001$) with heading date.

At seven marker–trait associations, the exotic alleles contributed to reduce days to heading up to 6.7 days (10.82% decrease, HEA2 effect at partially irrigated plots). In contrast, exotic alleles at five loci on 1H, 2H, 3H and 5H were found to have an effect of increasing heading date, with HEA4 on 2H ($P<0.05$) delaying heading by up to 2.1 days (3.5%) explaining 10.8% of the variation observed.

ii. Tiller number (TILL)

Ten chromosome regions were found significantly associated with number of tillers (TILL) across all chromosomes except 1H, predominantly for the marker main effect (Table 3.5). TILL2 (2H) showed the most significant effect ($P<0.001$) explaining 34.6% of the phenotypic variation observed for the marker main effect and reduced the number of tillers by 13.8% when the exotic alleles were present at the locus (20.9 fewer tillers). The exotic alleles were generally associated with diminished tillering, however at two of the QTL an increase in number of tillers was observed (TILL1 on 1H for the marker main effect (10.6% increase, 15.3 tillers) and TILL7 on 4H in fully irrigated (9.1% increase, 14.1 tillers)). Although a considerable proportion of the phenotypic variation was explained by the peak marker at TILL1 (24.7%), only a very small amount of this was found in the interaction with the treatment ($R^2<0.01\%$).

Table 3.5 List of QTLs identified for the developmental traits heading date (HEA) and number of tillers (TILL)

Trait	QTL	Marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP (%)[7]	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						M	MxT		[*Hsp*]	[*Hv*]				
HEA	HEA1	11_20502	1H	4.1–5.0	MxT	ns	*	D	61.6	61.2	0.65	0.28*	0.0–5.0	5.00
								FI	62.5	62.1	0.62			
								PI	62.2	61.3	1.49			
	HEA2	SCRI_RS_144545	2H	10.5–26.1	M	***	***	n/a	55.9	62.1	-9.99	51.12	0.0–41.9	41.90
		SCRI_RS_144545	2H	10.5–26.1	MxT	***	***	D	56.1	61.7	-8.96	0.47*	0.0–43.7	43.70
								FI	56.2	62.6	-10.17			
								PI	55.3	62.0	-10.82			
	HEA3	SCRI_RS_170235	2H	56.2–60.7	M	*	ns	n/a	60.0	62.1	-3.31	11.80	56.2–60.7	4.50
	HEA4	12_30897	2H	80.0–80.9	M	*	ns	n/a	63.4	61.3	3.48	10.79	79.4–86.0	6.60
	HEA5	11_21125	2H	120.8–131.9	MxT	ns	*	D	62.4	61.2	1.87	0.26*	86.0–140.8	54.80
								FI	63.4	62.1	2.13			
								PI	63.4	61.4	3.32			
	HEA6	SCRI_RS_189757	3H	23.9–35.1	MxT	ns	*	D	61.9	61.2	1.04	0.26*	23.9–35.1	11.20
								FI	63.7	62.1	2.63			
								PI	62.4	61.4	1.60			
	HEA7	11_10606	4H	60.8–64.3	M	*	ns	n/a	59.5	62.0	-4.11	13.56	60.8–64.3	3.50
	HEA8	SCRI_RS_179438	4H	73.5–76.3	M	*	ns	n/a	59.5	62.0	-4.01	12.66	73.5–76.3	2.80
	HEA9	SCRI_RS_206565	5H	96.6–106.6	MxT	ns	**	D	60.5	61.4	-1.39	0.31*	95.5–106.6	11.10
								FI	61.4	62.3	-1.47			
								PI	60.2	61.7	-2.50			
	HEA10	SCRI_RS_214550	5H	120.2–121.7	MxT	ns	*	D	62.4	61.2	1.93	0.17*	120.2–121.7	1.50
								FI	63.8	62.1	2.74			
								PI	62.3	61.5	1.43			
	HEA11	11_20247	7H	106.3–121.8	M	***	ns	n/a	56.1	62.1	-9.60	46.81	81.5–128.3	46.80
	HEA12	SCRI_RS_181575	7H	124.6–127.2	MxT	**	*	D	57.6	61.6	-6.49	0.20*	124.6–127.2	2.60
								FI	57.8	62.5	-7.60			
								PI	57.2	61.8	-7.53			

Table 3.5 Continued

Trait	QTL	Marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP (%)[7]	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						M	MxT		[*Hsp*]	[*Hv*]				
TILL	TILL1	SCRI_RS_170235	2H	56.2–60.7	M	**	ns	n/a	160.59	145.25	10.56	24.72	56.2–62.5	6.30
	TILL2	SCRI_RS_171032	2H	79.4–80.0	M	***	ns	n/a	130.43	151.31	-13.80	34.65	73.5–120.8	47.30
	TILL3	SCRI_RS_141166	3H	51.2–51.3	MxT	ns	*	D	126.33	121.19	4.25	0.00*	51.2–51.3	0.10
								FI	144.28	158.06	-8.72			
								PI	166.39	166.65	-0.15			
	TILL4	SCRI_RS_183659	3H	62.7–69.0	M	**	ns	n/a	128.63	150.72	-14.65	29.89	58.6–73.0	
	TILL5	SCRI_RS_119628	4H	17.8–19.9	MxT	ns	*	D	127.00	121.13	4.84	0.00*	17.8–19.9	2.10
								FI	145.90	157.94	-7.62			
								PI	165.98	166.68	-0.42			
	TILL6	SCRI_RS_222133	4H	49.9–51.4	M	*	ns	n/a	138.32	151.06	-8.43	15.97	49.9–51.4	1.50
	TILL7	SCRI_RS_148392	4H	67.6–73.5	MxT	ns	***	D	121.85	121.46	0.33	0.00*	67.6–73.5	5.90
								FI	168.28	154.21	9.12			
								PI	164.89	167.07	-1.31			
	TILL8	SCRI_RS_165569	5H	114.9–120.2	M	**	ns	n/a	115.60	149.60	-22.72	24.73	114.9–121.7	6.80
	TILL9	SCRI_RS_164037	6H	86.3–93.1	M	**	ns	n/a	115.60	149.60	-22.72	24.73	54.9–100.4	45.50
	TILL10	11_20847	7H	127.2–128.3	MxT	ns	*	D	115.50	122.23	-5.51	0.00*	127.2–128.3	1.10
								FI	161.01	156.66	2.78			
								PI	158.71	167.54	-5.27			

[1] Marker representing the block of markers defining the QTL peak
[2] Main effect region defined by the block of markers for the QTL peak
[3] Variation of the trait explained by the marker (M) or the marker–treatment interaction (MxT)
[4] Level of significance of the marker main effect (M) and the marker–treatment interaction (MxT). ***P<0.001,**P<0.01,*P<0.05, ns: not significant
[5] Treatment considered for the estimated means: drought (D), full irrigation (FI), partial irrigation (PI). Non applicable when means were estimated at the marker main effect level
[6] Estimated means (BLUEs) for each trait for the the donor ([*Hsp*]) and the recurrent parent ([*Hv*]) alleles at the peak QTL region
[7] Relative performance (RP) of the exotic parent alleles on the trait
[8] Variation of the trait explained by the marker and the marker–treatment interaction (*).Note that due to the linkage disequilibrium among the markers, R^2 values cannot be added together to give an estimate of the overall (%) variance explained.
[9] QTL significance region considering the blocks of markers flanking the QTL peak block
[10] Size of the QTL significance region

3.3.2.3 Agronomic traits

i. Dry Yield (DY)

A total of 15 marker–trait associations were found significant across all chromosomes for dry yield (DY), ten for marker main effect and five for the interaction with the treatment (Table 3.6). The most significant association (P<0.001) was found on chromosome 4H at the MxT level (DY9) where the peak marker explained 6.7% of the phenotypic variation. The marker main effect association for DY2 and DY3 on chromosome 2H accounted for the largest phenotypic variance among the significant associations (27.2% and 23.7% respectively). In general, the exotic alleles contributed to reduce yield. For example, exotic alleles at DY3 reduced yield by 16.4% (527.5 kg ha^{-1}) compared to the cultivated barley alleles at this loci. However, the exotic alleles did have a positive effect at a few marker–treatment interactions. Caesarea alleles at DY4 (3H), DY9 (4H) and DY15 (7H) improved yield performance in full irrigation. DY9 (4H) showed the most significant association (P<0.001) with exotic alleles increasing yield by 11.6%. Additionally, exotic alleles were found to slightly improve yield (2.7%) under drought conditions at DY8 loci on 4H, however a 9.2% reduction under full irrigation was also associated with the locus.

ii. Thousand grain weight (TGW)

Sixteen chromosome regions were significantly associated with thousand grain weight (TGW) across all chromosomes (Table 3.6), eight for the marker main effect, seven for the interaction with the treatment and one showing both level effects. TGW1 (1H), TGW10 (4H) and TGW12 (5H) explained the greatest amount of phenotypic variation observed at the marker main effect level (20.8%, 18.3% and 19% respectively). In general, the exotic alleles were found to improve this trait in comparison with the elite barley alleles. For example *Hsp* alleles at TGW12 (5H) increased TGW by 5.7% (2.6 g), having a more noticeable effect under drought (7.4% increase, i.e. 3.5 g). In contrast, at TGW1 (1H) the exotic alleles had the opposite effect, reducing TGW by 7.2% (3.3 g).

Table 3.6 List of QTLs identified for the agronomic traits dry yield (DY) and thousand grain weight (TGW)

Trait	QTL	Marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP (%)[7]	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						M	MxT		[*Hsp*]	[*Hv*]				
DY	DY1	SCRI_RS_144315	1H	85.8–90.2	M	*	ns	n/a	2903.4	3207.6	-9.49	10.05	85.8–90.2	4.40
	DY2	SCRI_RS_171032	2H	79.4–80.0	M	**	ns	n/a	2724.8	3224.1	-15.49	27.21	68.6–81.5	12.90
	DY3	SCRI_RS_158091	2H	107.9–118.7	M	**	ns	n/a	2682.5	3210.0	-16.43	23.66	106.4–119.8	13.40
	DY4	11_20858	3H	0.0–2.7	MxT	ns	**	D	2694.0	2756.2	-2.26	4.33*	0.0–2.7	2.70
								FI	3685.8	3168.6	16.32			
								PI	3622.8	3500.8	3.49			
	DY5	SCRI_RS_189757	3H	23.9–35.1	MxT	ns	*	D	2743.5	2752.5	-0.33	3.73*	23.9–43.1	19.20
								FI	2699.3	3241.8	-16.74			
								PI	3337.2	3521.8	-5.24			
	DY6	11_11002	3H	43.1–46.2	M	*	ns	n/a	2768.2	3199.8	-13.49	14.31	43.1–46.2	3.10
	DY7	SCRI_RS_220192	3H	61.8–62.5	M	*	ns	n/a	2807.7	3195.4	-12.13	10.83	61.8–69.0	7.20
	DY8	SCRI_RS_14857	3H	105.0–115.9	MxT	ns	*	D	2818.8	2744.1	2.72	3.00*	105.0–115.9	10.90
								FI	2937.5	3235.0	-9.20			
								PI	3531.9	3506.4	0.73			
	DY9	SCRI_RS_148392	4H	67.6–73.5	MxT	ns	***	D	2730.4	2757.6	-0.99	6.77*	49.9–76.3	26.40
								FI	3493.5	3129.2	11.64			
								PI	3504.1	3510.4	-0.18			
	DY10	SCRI_RS_175087	5H	30.4–31.3	M	*	ns	n/a	2740.2	3203.1	-14.45	17.11	30.4–40.0	9.60
	DY11	SCRI_RS_149088	5H	106.6–112.6	M	*	ns	n/a	2803.9	3195.8	-12.26	11.28	106.6–112.6	4.40
	DY12	11_21124	6H	54.9–57.2	M	*	ns	n/a	2839.4	3221.2	-11.85	18.05	54.9–72.9	18.00
	DY13	SCRI_RS_160602	7H	29.8–42.5	M	*	ns	n/a	2857.1	3229.8	-11.54	15.72	29.8–43.3	13.50
	DY14	SCRI_RS_200021	7H	84.6–89.5	M	*	ns	n/a	2883.1	3211.8	-10.23	12.45	84.6–89.5	4.90
	DY15	11_20847	7H	127.2–128.3	MxT	ns	*	D	2654.7	2763.1	-3.92	3.85*	127.2–128.3	1.10
								FI	3433.7	3178.0	8.05			
								PI	3340.7	3528.6	-5.32			
TGW	TGW1	11_11367	1H	61.8–66.3	M	**	ns	n/a	42.91	46.22	-7.16	20.84	61.5–76.8	15.30
	TGW2	SCRI_RS_192730	1H	100.9–116.3	MxT	ns	*	D	48.16	47.75	0.86	1.69*	100.9–116.3	15.40
								FI	46.21	44.04	4.92			
								PI	46.48	45.33	2.54			
	TGW3	SCRI_RS_147371	2H	26.8–28.7	MxT	ns	*	D	52.38	47.66	9.89	1.27*	26.8–28.7	1.90
								FI	46.17	44.26	4.31			
								PI	49.94	45.36	10.09			19.20

Table 3.6 Continued

Trait	QTL	Marker[1]	Chr	Main effect region (cM)[2]	Effect[3]	p-value[4]		Treat[5]	Estimated means[6]		*Hsp* RP (%)[7]	R^2 (%)[8]	QTL significance region (cM)[9]	QTL size (cM)[10]
						M	MxT		[*Hsp*]	[*Hv*]				
TGW (cont)	TGW4	11_20173	2H	38.1–40.8	M	*	ns	n/a	48.49	45.57	6.40	15.44	28.7–41.9	13.20
	TGW5	SCRI_RS_144891	2H	55.5–56.2	M	*	ns	n/a	47.55	45.53	4.45	10.56	55.5–56.2	0.70
	TGW6	12_30674	2H	68.6–68.6	MxT	ns	**	D	47.36	47.87	-1.07	2.10*	68.6–73.5	4.90
								FI	45.56	44.15	3.19			
								PI	45.90	45.42	1.05			
	TGW7	11_20252	3H	2.7–3.1	M	*	ns	n/a	48.26	45.60	5.84	12.26	2.7–3.1	0.40
	TGW8	11_10559	3H	20.4–23.9	M	*	ns	n/a	48.27	45.60	5.85	12.35	20.4–23.9	3.50
	TGW9	SCRI_RS_150051	4H	0.0–17.8	MxT	ns	*	D	47.09	47.95	-1.79	1.46*	0.0–17.8	17.80
								FI	44.70	44.27	0.99			
								PI	45.89	45.40	1.08			
	TGW10	SCRI_RS_183399	4H	42.1–45.7	M	**	ns	n/a	47.56	45.34	4.91	18.28	40.0–49.9	9.90
	TGW11	SCRI_RS_179438	4H	73.5–76.3	M	*	ns	n/a	47.88	45.55	5.10	11.88	73.5–76.3	2.80
	TGW12	SCRI_RS_237352	5H	95.5–96.6	M	**	*	n/a	48.00	45.43	5.66	18.97	95.5–106.6	11.10
		SCRI_RS_237352	5H	95.5–96.6	MxT	**	*	D	50.70	47.20	7.42	1.75*	87.4–96.6	9.20
								FI	45.97	44.00	4.48			
								PI	47.33	45.10	4.96			
	TGW13	SCRI_RS_214550	5H	120.2–121.7	M	*	ns	n/a	49.23	45.63	7.89	16.48	120.2–121.7	1.50
	TGW14	SCRI_RS_225193	6H	69.3–72.9	MxT	ns	*	D	48.45	47.70	1.57	1.21*	69.3–72.9	3.60
								FI	43.98	44.40	-0.95			
								PI	46.47	45.33	2.51			
	TGE15	SCRI_RS_151280	6H	117.5–126.6	MxT	ns	**	D	49.33	47.32	4.25	2.37*	117.5–126.6	9.10
								FI	44.65	44.24	0.93			
								PI	46.61	45.12	3.30			
	TGW16	11_20247	7H	106.3–121.8	MxT	ns	*	D	50.63	47.59	6.38	1.59*	106.3–121.8	15.50
								FI	44.93	44.30	1.43			
								PI	46.89	45.38	3.32			

[1] Marker representing the block of markers defining the QTL peak
[2] Main effect region defined by the block of markers for the QTL peak
[3] Variation of the trait explained by the marker (M) or the marker–treatment interaction (MxT)
[4] Level of significance of the marker main effect (M) and the marker–treatment interaction (MxT). ***P<0.001,**P<0.01,*P<0.05, ns: not significant
[5] Treatment considered for the estimated means: drought (D), full irrigation (FI), partial irrigation (PI). Non applicable when means were estimated at the marker main effect level
[6] Estimated means (BLUEs) for each trait for the the donor ([*Hsp*]) and the recurrent parent ([*Hv*]) alleles at the peak QTL region

[7] Relative performance (RP) of the exotic parent alleles on the trait.
[8] Variation of the trait explained by the marker and the marker–treatment interaction (*). Note that due to the linkage disequilibrium among the markers, R^2 values cannot be added together to give an estimate of the overall (%) variance explained.
[9] QTL significance region considering the blocks of markers flanking the QTL peak block
[10] Size of the QTL significance region

3.3.3 Pleiotropic effects associated with plant development QTLs

As expected from the numerous correlations found between measurable characteristics defining the morphology, development and agronomic performance of the RCSLs (Chapter 2), some chromosome regions were found to be influencing more than one trait. Common QTL for several traits were found on all chromosomes. Closely related traits such as peduncle length and extrusion or seed area and seed length were generally associated with the same regions (Appendix 14). However, in some cases loci associated with secondary traits, such as heading date (HEA), were found to be influencing morphological traits such as collar height (COL) and number of tillers (TILL) as well as agronomic traits like dry yield (DY) and thousand grain weight (TGW). Known genes associated with plant flowering time and plant height were located on the RCSLs genetic map to determine whether they are responsible for the effect of some of the major QTLs identified.

3.3.3.1 Heading date known genes effect

The strongest associations (P<0.001) for days to heading were found on chromosomes 2H (HEA2) and 7H (HEA11) (Appendix 14). The peak marker association at HEA2 on 2H (10.5 cM to 26.1 cM) was defined by the marker block represented by the SNP marker SCRI_RS_144545 which was found linked to the transcript MLOC_81154.1 (contig_94710) identified for the photoperiod response gene *Ppd-H1* (Laurie *et al.*, 1995; Turner *et al.*, 2005). The locus was also found to be associated with BY1 for the QTL main effect (P<0.001) as well as COL3 (P<0.01), SdL2, SdA2 and PdL2 (P<0.05) in the marker–treatment interaction effect. Here the exotic alleles reduced time to heading by six days, decreased biomass production (7% less) and had shorter heights (up to 11.7 cm shorter plant under full irrigation) but were associated with longer peduncles and larger seeds, particularly under drought (3.9 cm and 0.82 mm^2 estimated increase respectively). Chromosome regions associated with TGW (TGW3 and TGW4) were found in the vicinity of HEA2. Here the exotic alleles contribute to improving the trait.

Similar effect was observed for a cluster of QTLs located on chromosome 7H sharing a peak marker region represented by SCRI_RS_200021 (from 84.6cM to 89.5cM). Exotic alleles were found to affect plant phenology and performance by decreasing time to heading by six days (HEA11) and reduce yield by 10.2% (DY14) while lengthening the plant peduncles PdE9 and PdL12 (4.4 cm and 4.9 cm respectively). The *earliness per se* gene *eps7L* (Laurie *et al.*, 1995) in this location was considered as a good candidate to explain the effect on heading. Also, the

photoperiod response gene *HvCO1* (Griffiths *et al.*, 2003) was found to map in the vicinity of HEA11.

Other QTL effects were detected for heading date main effect showing pleiotropic effects on agronomic traits but with a weaker association. For example, in the centromeric region of chromosome 2H, the peak marker block (represented by SCRI_RS_170235) for HEA3 ($P<0.05$) was found associated with COL4, EAR2, SdW2 and TILL1. Wild barley alleles contributed to shorten the time to reach heading (2.1 days) reduce collar height (8.7 cm), lengthen the spike (0.9 cm) and increase the seed width (0.1 mm) and number of tillers (15.3 more tillers). The gene *HvCEN* associated with flowering time (Comadran *et al.*, 2012) was regarded as a good candidate to explain the phenotypic variation found for these QTLs since the SNP marker identified co-segregating with this gene (12_30265) was found polymorphic for the group of RCSLs and located within the peak marker block for HEA3.

Since the transition from vegetative to reproductive state in barley depends not only on day length (photoperiod response) but also on low temperatures requirement (vernalisation response), the position of the main genes associated with the latter were also determined (Cuesta-Marcos *et al.*, 2010). Vrn-H1, Vrn-H2 and Vrn-H3 were associated with the genetic positions of the RCSLs marker blocks represented by SCRI_RS_133602 on 5H (125.5cM to 136.4cM), 11_10611 on 4H (111.3cM to 115.2cM) and SCRI_RS_160602 on 7H (29.8cM to 42.5cM) respectively. None of these blocks of markers conformed to the peak region for any of the associations found for heading date; nevertheless, a slight delay ($P<0.05$) on flowering attributed to the exotic alleles was found close to the Vrn-H1 region at HEA10 (102.2 cM to 121.7 cM on 5H). Additionally, although the Vrn-H3 region was not found associated with heading, a main association with DY13 was observed for the marker block linked to this gene where the exotic alleles reduced yield about 11.5% (R^2=15.7%).

3.3.3.2 Plant height known genes effect

Highly significant associations ($P<0.001$) with plant height were detected for COL10 (3H), COL14 (5H), COL16 (6H), where the wild barley alleles contributed considerably to increase the height of plant collar. The main effect of the peak marker block for the region explained up to 38.1% (COL16) of the phenotypic variation observed and increased height in 27.1 cm (COL10) when compared to the effect of the barley cultivar alleles.

The region on 3H spanning 7 significant marker blocks from 103.8 cM to 148.2 cM was associated with the *sdw1/denso* semi-dwarf gene locus on the groups of markers represented by

SCRI_RS_14857 (105.5 cM to 115.9 cM) for which the functional gene of the *sdw1/denso* locus, *HvGA20ox*$_2$ (MLOC_56462) (Jia *et al.*, 2015), was found assigned to the region. The wild barley alleles at the locus were not only associated with increased plant collar height, but also the estimated seed width in 0.14 mm (SdW6). In contrast, pleiotropic effects on harvest index (HI8 and HI9) and dry yield (DY8) were identified for this chromosome region. A decrease from 42.4% to 36.5% of the estimated proportion of economic yield per unit of biomass was associated to the main effect of exotic alleles at HI9 (84.4 cM to 146.4 cM). Also a 9.2% reduction in estimated yield was found for the exotic alleles at DY8 (105.0 cM to 115.9 cM) in the irrigated treatment, however, an slight increase of 2.7% estimated yield was detected for the exotic alleles effect at DY8 under drought.

No known gene candidates were found to explain the variation estimated for plant height on COL14 (5H, 140.1 cM to 144.7 cM) and COL16 (6H, 57.2 cM to 86.3 cM) although previous studies have identified plant height QTLs around the same chromosome regions (Tinker *et al.*, 1995; Talamè *et al.*, 2004; Lakew *et al.*, 2013). The strong main marker effect on these QTLs was found associated with possible pleiotropic effects on morphological traits such as the length of the peduncle (PdL6, PdL7, PdE5 and PdE6) for the chromosome region found on chromosome 5H (COL14) and the length of the spike (EAR9 and EAR10) for the region on 6H (COL16). For all these associations, the exotic alleles contributed to increase estimated plant height (14.5 cm for COL14, 21.5 cm for COL16), length of the peduncle (up to 5.6 cm increase on PdE6) and spike (up to 1.0 cm increase on EAR9). However, COL14 and COL16 were also related to decreased harvest index (HI12, HI13 and HI16) for the exotic alleles (from 42.5% to 32.3% decrease on the estimated harvest index at HI16). Also, the increase in height associated with the exotic alleles at COL16 was found to reduce the number of tillers (TILL8 and TILL9) and yield (DY12) but increase biomass (BY8) and TGW (TGW14) (Appendix 14).

3.3.4 From QTL to candidate gene using the genetic and the physical map

Thirty seven genomic regions associated with a group of traits were selected based on the main effect found on the estimated phenotypic variation for the RCSLs and also for the potential of the wild barley alleles to improve economically important traits (Appendix 15).

Heading (HEA) and height (COL) were used to verify the association of the major QTLs with known genes in the barley new genome assembly (IBSC, 2016). Thousand grain weight (TGW), seed width (SdW) and seed length (SdL) QTLs were selected due to the transgressive segregation observed in the RCSLs for TGW (see section 2.4.2.3) and the fact that exotic alleles at these associations significantly improved the performance of the trait (14 out of 16 QTLs, 7

positive associations at the marker main effect level). The annotated genes that could potentially be associated with candidate genes whose function could have contributed to increase the seed plumpness, and so its weight, were investigated.

Also, a 3.2 cM genomic region on top of chromosome 1H (0 to 3.2 cM) between markers 12_30969 and 12_30715 was targeted due to its association with the glossy spike phenotype and denoted as GLS1. This trait was scored in five RCSLs (see section 2.4.2.1) sharing a unique exotic introgression in the region defined by these two markers that was not present in any of the other lines.

3.3.4.1 Heading and height

Six chromosome regions were found significantly associated with heading date (HEA) at the marker main effect level (Appendix 13). HEA2 (2H) had the highest significant effect and the candidate photoperiod response gene *Ppd-H1* was selected from the literature to explain the phenotypic variation for the loci (Turner *et al.*, 2005). Despite the large number of annotated genes (914) defined for the 46.9 Mbp region, HORVU2Hr1G013400.32 was found within the 15.4 Mbp region defined for the peak block of markers for HEA2(2H) as the gene corresponding to *Ppd-H1* which is described as a "pseudo-response regulator 7" in the new assembly.

Similar process was conducted to find the *HvGA20ox*$_2$ gene described as the functional gene of the *sdw1/denso* locus (Jia *et al.*, 2015) which was identified as a potential candidate to explain the phenotypic variation associated to COL10(3H). Only by using the GO annotations and descriptors of the gene, it was possible to identify HORVU3Hr1G090980.3 as the functional gene of the locus within the 60.7 Mbp region with 1137 genes annotated.

On the other hand, none of the annotated genes for the smallest chromosome regions associated ($P<0.05$) with heading, HEA8(4H), and height, COL20(7H), were found as good candidates to explain the variation of the traits based on their ontology and description.

3.3.4.2 TGW and seed characteristics

The genomic regions targeted for TGW, SdW, SdL and GLS ranged from 0.3 cM (SdW3) to 56.9 cM (SdL7). The physical distance between flanking markers went from few base pairs (660,247bp SdW3) to more than 529 Mbp (SdL7). The number of genes annotated for different regions varied considerably, reaching up to 3251 for SdW7. In contrast only 4 annotated genes

were determined for COL20. Also, regions of similar genetic distance between flanking markers such as SdW1 on 1H (52.3 cM to 59.1 cM) and SdW2 on 2H (53.8 cM to 59.9 cM) were found to differ greatly for the number of genes annotated (390 genes SdW1 and 2084 genes SdW2). However, two relatively small chromosome regions (on 5H and 3H) were targeted and characterised due to the overlapped main effect of the markers at the loci for seed weight (TGW) and width (SdW) where the wild barley haplotype was found to improve both estimated trait means. The chromosome region shared between SdW7 and TGW10 on 4H (34.3 to 54 cM) showed the same positive association for the two traits. However, due to the large number of annotated genes found for the region (2001 pseudomolecules for SdW7) no further investigation was proposed.

i. SdW11 – TGW13 (5H)

The 1.5 cM genomic region on 5H at 120.2 cM (associated with SdW11 and TGW13) was used as a proof of concept to identify putative candidate genes that could play a role increasing seed plumpness and consequently seed weight (Appendix 16, CD-ROM). Briefly, a 2.9 Mbp region was defined between the two flanking markers (SCRI_RS_214550 and SCRI_RS_174710) for which 51 annotated genes were found using the new barley genome assembly (IBSC, 2016), of which 27 high confidence genes had been identified in the previous barley genome assembly (IBSC, 2012). Identification of putative candidates was based on the descriptors and ontology terms for these genes. The MLOC_71738.1 (contig_60402) was identified as 'an interesting candidate' due to its role as a "sugar transporter" and its annotation terms related to transmembrane transporter activity. According to the new genome assembly (IBSC, 2016), the 3301 bp of this MLOC were found to map within the HORVU5Hr1G093350.11 transcript (4602 bp) which has been annotated as "Solute carrier family 22 member 1". Furthermore, the BLAST analysis using the 2019 bp DNA sequence defined for MLOC_71738.1 in the morexGenes database (https://ics.hutton.ac.uk/barleyGenes/) identified another transcript MLOC_76072 (contig_7003) with 89.5% similarity (E-value: 4.6E-44) which is located in the same genomic region on 5H and has been described as related to transmembrane transport in molecular and biological processes. In this case, the physical position of the MLOC_76072.2 overlaps with the HORVU5Hr1G093390.2 transcript from the new assembly, also described as a "Solute carrier family 22 member 1". Both MLOC showed alignments with a homologous sequence in rice on chromosome 3 described as a "transporter family protein" (LOC_Os03g43720).

ii. SdW5- TGW8 (3H)

In this case a 5.1 cM region on chromosome 3H corresponding to SdW5 (from 20.4 cM to 25.5 cM) was selected to explore the annotations of the genes in this region (Appendix 17, CD-ROM). The 3.6 Mbp of the physical map were found to contain 92 annotated genes according to the new assembly (51 high confidence genes in the previous 2012 assembly). The transcript HORVU3Hr1G010530.2 (corresponding to MLOC_61457.1) was found as an 'interesting candidate' to explain the phenotypic variance associated with this chromosome region putatively associated with increased seed width and weight. The GO annotation for this gene (GO:0019904) describes a "protein domain specific binding" and it is predicted to encode a 14-3-3 protein; these have been found to be important regulatory proteins involved in carbohydrate metabolism processes associated with starch accumulation during the development of grain in wheat (Yu *et al.*, 2016), rice (Zhang *et al.*, 2014) and barley (Alexander and Morris, 2006).

Another gene in this region which could be a potential candidate was HORVU3Hr1G010570.2, or MLOC_74351.2 due to its function as an oligopeptide transporter (PTR2 family protein), however the role of this transporter in the grain filling process in cereal crops or model plants was unclear from the literature.

iii. GLS1 (1H)

The telomeric region on top of 1H between markers 12_30969 (0 cM) and 12_30715 (3.2 cM) was found to be 584,212 bp in size with 20 annotated genes (27 MLOCs on the previous 2012 assembly) (Appendix 18, CD-ROM). A putative candidate (HORVU1Hr1G000320.14) was identified within this region based on its predicted function as esterase/lipase/thioesterase family protein. Thioesterases appear to be involved in the plant cuticular wax biosynthesis by modifying acyl chains to generate fatty acids that are transported through the plastidial membrane (Shepherd and Griffiths, 2006).

Other interesting candidates were found close to the target region. HORVU1Hr1G000150.13, with "O-acyltransferase (WSD1-like) family protein" predicted function, and HORVU1Hr1G000190.3 "Fatty acyl-CoA reductase 1" were located at 551,441 bp and 453,531 bp from the position for the marker 12_30969 on top of chromosome 1H. The gene annotation for the latter has been shown to be involved in the wax biosynthesis pathway in Arabidopsis (Rowland *et al.*, 2006) and is also one of a cluster of genes (*Cer-cqu*) on barley chromosome 2H (Schneider *et al.*, 2016) and therefore a potentially interesting candidate for further study.

3.4 Discussion

In this chapter, the genotypic characterisation of a set of 28 barley Recombinant Chromosome Substitution Lines (RCSLs) was used in a marker–trait association analysis to define chromosome regions accounting for significant phenotypic variation in thirteen traits measured in field trials (2013 and 2014) under different water regimes. A total of 161 marker–trait associations were identified either for the marker main effect (major QTLs) or in response to the water treatment (minor QTLs). Many of these associations were confirmed from previous studies in the RCSLs as well as in other AB-populations and explained the major phenotypic differential traits between wild and domesticated barley. In addition, some chromosome regions were found where the exotic genome was positively associated with secondary traits that could potentially impact on crop production.

3.4.1 Marker–trait associations were detected using a group of 28 RCSLs

3.4.1.1 Genome coverage of the wild barley accession in a set of 28 RCSLs

Complete genome coverage of the wild barley accession was achieved with a representative set of 28 RCSLs, each of which showed a mean substitution ratio of exotic alleles of 13.4%, similar to the 13% average defined for the entire population in the GCP project (2010, unpublished) using the BOPA1 SNP platform (Close *et al.*, 2009). Although there is a loss of mapping resolution from using a subset of lines from the original population, the greater number of polymorphic markers (1848 SNPs), defined from the 9K SNP chip for barley (Comadran *et al.*, 2012), has considerably improved the estimation of the extent and overlap of the substituted segments from Caesarea 26-24, including 1083 extra SNP polymorphisms to the previous genotypic characterisation. Therefore this subset of unique lines provides a more precise coverage of the wild genome with a higher degree of marker saturation, and is therefore more amenable to QTL and fine mapping studies.

3.4.1.2 Marker–trait association analysis using a hierarchical mixed model approach

Because the study was based on a subset of selected lines from the original population (Matus *et al.*, 2003), conventional methods for QTL mapping in bi-parental populations (F_2, RILs, BC, etc.) such as interval mapping (IM) or composite interval mapping (CIM) were unsuitable (Hackett, 2002). Although other groups working with similar populations have developed methods such as the S42ILs developed by Klaus Pillen's group (Schmalenbach *et al.*, 2008), these were also found to be inappropriate, mainly since the method locates QTLs to a specific

chromosomal interval represented by one or very few ILs carrying uniquely the target segment making the ILs performance significantly different from the background parent. Because in this population, each of the RCSLs harbour on average 6 exotic introgression, which vary in size and overlaps, the REML single locus analysis using a hierarchical mixed model was shown to be a more effective and straightforward approach to identify meaningful marker–trait associations. Not only were major loci controlling main plant developmental traits, such as plant height and heading date, identified and verified from the literature, but also new chromosome regions for which the wild barley genome could contribute favourable alleles to improve the crop performance were found.

Only by breaking up these introgressions, through new backcrossing strategies, could QTLs be located to smaller genomic regions and candidates genes for specific traits identified. In addition, approaches using REML forward selection described by Bauer *et al.* (2009) could potentially reduce the number of significant marker–trait associations in a step wise analysis, improving the power of the QTL mapping. However in this case a larger group of lines would be necessary. Hence, for the scope of this present study, the REML single-locus analysis was found to be the most appropriate and efficient approach to detect marker–trait associations in a small group of lines.

3.4.2 Major QTLs underlie the variation found for morphological and developmental traits discriminating wild and domesticated barleys

The RCSLs wild parent, Caesarea 26-24, not only possesses morphological traits undesirable from a breeding perspective, such as those related with seed dispersal, but also the ancestral winter growth habit that allows adjusting the flowering time to the favourable season in response to environmental signals (Karsai *et al.*, 2004). This phenotype is in contrast to the spring cv. Harrington that has been selected for long growing seasons (irresponsive to photoperiod) and also carries genes introduced through breeding to maximise productivity in high-input systems. The segregation of genes governing plant life cycle and morphological features discriminating wild barley from its cultivated form are illustrated with three examples: flowering, height and domestication QTLs.

3.4.2.1 Flowering time genes

The exotic alleles conferred earliness to the majority of the associations found for heading date; however, the loci associated with genes controlling the vernalisation response were not found to delay flowering time significantly as we would have expected from a spring sowing trial of

these lines (Fig. 3.5). Unfortunately, no previous characterisation of the RCSLs phenology can be used to confirm this assumption.

Figure 3.5 Caesarea 26-24 experimental field plot: winter vs spring sown.

The picture was taken the 18th June 2013 (by Allan Booth): winter-sown plants on the left produced ears in the beginning of June. In contrast, plants on the right were sown in spring and eventually produced a single ear per plant in late July

The main determinant of long day photoperiod response locus (*Ppd-H1*) and an earliness *per se* locus (*eps7L*) (Laurie *et al.*, 1995) were identified as good candidate genes for the major QTLs associated with heading date on chromosomes 2H(HEA2) and 7H(HEA11). Both loci exerted pleiotropic effects on yield related QTLs co-locating in the same region. Biomass accumulation, plant height and yield were reduced as a consequence of the limitation in vegetative growth, which contrasted with the favourable effect on grain weight. Similar associations have been reported in other bi-parental AB-populations using germplasm or landraces from the Fertile Crescent as donor genome, particularly those corresponding to the *Ppd-H1* locus (Baum *et al.*, 2003; Talamè *et al.*, 2004; Li *et al.*, 2006; Wang *et al.*, 2010), but also for *eps7L* locus (Schmalenbach *et al.*, 2009; Schnaithmann and Pillen, 2012). Nevertheless, it should be noted that the large effect of these QTLs could have partially masked the effect of other flowering genes found in the proximity of these loci such as *HvCEN* on 2H (Comadran *et al.*, 2012) and the photoperiod response gene *HvCO1* on 7H (Griffiths *et al.*, 2003; Campoli *et al.*, 2012).

Only *HvCEN* has been found as the gene determining the observed variation in flowering time for *eps2*, one of the *earliness per se* loci identified in barley (Laurie *et al.*, 1995). The natural variation of the locus revealed its contribution to the adaptation of barley to higher latitudes (Comadran *et al.*, 2012). However, little is known about the other earliness loci. The dissection of the 46.8 cM region corresponding to the *eps7L* locus on the long arm of chromosome 7H using a group of NILs could provide the means to fine map the QTL and identify possible candidates genes. In addition, new backcrosses would also allow the relationship between *esp7L* locus and the proximal *HvCO1* gene to be determined and their individual effect identified. Finally, this investigation could provide a new allelic variant to fine tune flowering time in spring barley cultivars with improved adaptation to specific environments.

3.4.2.2 Plant height

Breeding programmes have successfully optimised plant height through the introduction of different semi-dwarfing genes in particular the *sdw1/denso* locus in barley (Kuczyńska *et al.*, 2013). These genes have not only reduced lodging but also boosted harvest index by increasing the allocation of assimilates for grain production (Hedden, 2003).

One of the major QTLs for height in this study (COL10) was found in the region of the *sdw1/denso* locus on chromosome 3H revealing the presence of the *denso* mutation in cv. Harrington background. Here, the Caesarea 26-24 alleles increased estimated collar height by 27.1 cm with the subsequent negative impacts on harvest index observed. Interestingly, a minor QTL for yield (DY8) was co-located at the loci where the exotic alleles reduced predicted yield by 9.2%, which under drought actually resulted in a slight increase of 2.7% yield. This suggests that increased height associated with the exotic alleles at the *sdw1/denso* locus could have been beneficial for grain production under drought. Although this assumption agrees with the observations by Baum *et al.* (2003) using a group of RILs derived from a cross between a high yielding Syrian landrace (cv. Arta) and a wild accession (*Hordeum spontaneum* 41-1), it could not be confirmed with evidence from previous studies with these RCSLs.

Additionally, several other loci controlling plant height and crop performance were identified across the genome and confirmed in other AB-populations using unadapted germplasm (Baum *et al.*, 2003; Pillen, Zacharias and Léon, 2003; Talamè *et al.*, 2004; Li *et al.*, 2006; Gyenis *et al.*, 2007). Only a few examples were found where height was reduced. For example, on top of chromosome 6H the co-location of two minor QTLs where the exotic alleles reduced height (COL15), conferring a positive effect on harvest index (HI14), especially under irrigation, was observed. This association could not be confirmed from previous studies and generally, the

decreased height was a consequence of the pleiotropic effects of major flowering QTLs where the exotic alleles conferred earliness, reducing vegetative growth and so limiting height (see section 3.3.3).

Though the generalised contribution of the exotic genome to increase plant height at major QTLs detected for the trait might not seem interesting for detecting favourable alleles for the crop, these loci reveal chromosome regions of the recurrent parent that should be retained, working as "knock outs" in future breeding programmes (Matus *et al.*, 2003; Inostroza *et al.*, 2009).

3.4.2.3 Domestication traits

In the development of the RCSL population, Matus *et al.* (2003) reported that, despite most of the lines maintained a reasonable agronomic potential, some domestication-related traits such as seed and awn retention and shattering were still present in the population. In the present study not only these morphological traits associated with wild barley seed dispersal were found, but also the glossy spike phenotype that has not been reported before for this population and that might constitute an adaptive trait of the Caesarea 26-24 to the droughted conditions of its natural habitat (Shepherd and Griffiths, 2006).

i. Threshability locus (1H)

Awn retention in the seed is an adaptive mechanism for seed dispersal in wild barley, but undesirable for the crop since it obstructs the grain threshability process. This phenotype was observed in one of the RCSLs (OSU015) and associated to a 5 cM chromosome region on 1H (95.9 cM to 100.9 cM) confirming the results obtained from the barley wide cross cv. Scarlett × ISR 42-8 (Schmalenbach *et al.*, 2011). Here, the authors mapped the *thresh-1* locus within a 4.3 cM region using a high-resolution mapping population (91 S42IL-HR) derived from 73 introgression lines from the original population (S42IL) and proposed genes potentially involved on the synthesis of components strengthening the cell wall composition of the spike such as a polygalacturonase encoding gene and a cellulose synthase like family C (CSLC1).

ii. Glossy spike locus (1H)

The GLS*1* locus associated with the wild barley glossy spike phenotype and mapped on top of chromosome 1H (0-3.2cM) seem to affect the synthesis and deposition of epicuticular waxes,

particularly in the spike. This locus could correspond to the *Cer-yy* reported by Lundqvist and von Wettstein-Knowles (1982), for which no candidate genes have been defined yet. The phenotype observed seems to relate to a depletion of β-diketones, the biosynthesis of which, is directly related to the glaucous phenotype (Zhang *et al.*, 2015). Because of the role of epicuticular waxes in the protection of plant surfaces, GLS*1* was identified as a good target to investigate further and find potential candidates for improving barley adaptability to drought prone environments. Three good candidates were identified for the locus due to their predicted role in the metabolic processes related to wax biosynthesis pathway. Recently, Schneider et al. (2016) unravel the function of a cluster of three genes mapped on barley chromosome 2H (*Cer-cqu*) regulating the biosynthesis of cuticular wax components using a set of barley cultivars, NILs and mutants. A similar approach could be undertaken to fine-map GLS*1* and determine the function of the candidate genes identified in the formation of barley cuticle waxes.

3.4.3 Contribution of Caesarea 26-24 to improve the agronomic performance of the crop

Despite Caesarea 26-24 genome diminishing general performance of cv. Harrington as it was concluded in previous studies (Matus *et al.*, 2003; Inostroza *et al.*, 2009; del Pozo *et al.*, 2012), it seems to contribute favourable alleles for enhanced grain traits that could potentially improve crop yield performance.

Genetic gains on yield during the Green Revolution were associated with the introduction of new crop varieties carrying genes that reduced plant stature to an optimum. The repartition of assimilates in shorter semi-dwarf plants favoured larger grain production in terms of increased number of grains per unit land area. However, this gain in grain yield was coupled with slight reductions of individual grain weight and size (Hedden, 2003; Kuczyńska *et al.*, 2013). Therefore, in order to achieve further genetic gains on yield while maintaining high harvest indices, breeding efforts should focus on alternative secondary traits putatively related with higher yield potential but with higher heritability than yield itself such as grain size and weight (Araus *et al.*, 2008). In this regard, understanding the mechanisms involved in source and sink interactions during the post-anthesis phase, i.e. those regulating the partition of carbon assimilates produced during the pre-anthesis period into the sink organs or grains, becomes particularly important for identifying candidate genes in the target regions that might contribute to increase the sink strength and so grain size and weight (Coventry *et al.*, 2003; Araus *et al.*, 2008).

In this context, major QTLs on chromosome regions where the exotic alleles favoured size of individual grains showing concomitant effects on increased grain weight (TGW) were targeted for further investigation. Two of these major associations were confirmed with the QTLs

reported for TGW in the GCP project (2010, unpublished), however these loci were discarded for further investigations since they appeared to be related with indirect effects of main developmental loci influencing flowering time in barley. As explained by Coventry *et al.*, (2003), adjustments in plant cycle will determine the conditions for the pre-anthesis and post-anthesis periods, modulating the source and sink interactions that will influence grain size and weight at the end of the growing season (see section 3.4.2.1), therefore, in order to improve these traits only non-developmental loci with stable effects on grain size and weight would be good candidates for a marker assisted selection strategy to improve the traits.

Two loci following these premises on chromosomes 5H and 3H were identified as good candidates to investigate further by screening the functional annotations of genes within the intervals of the QTLs. Both regions seem independent of major developmental loci in the RCSLs and some candidate genes were identified as being supposedly involved in the transport, utilisation and storage of carbon assimilates during the complex process of grain development in the post-anthesis period (Thiel, 2014).

The chromosome region on 5H contains two candidate genes involved in the trans-membrane transport of sugar. During grain filling the synthesis of starch depends on the supply of sucrose and other compounds transported into the seed endosperm from maternal tissue (Sivitz *et al.*, 2005; Thiel, 2014), therefore this process could potentially define sink strength of the crop. In fact, the overexpression of the barley transmembrane sucrose transporter *HvSUT1* was found to increase the sink capacity of the grains, leading to increments on grain weight (Weichert *et al.*, 2010). Homologous genes of these two candidates were identified in rice but no evidences of their role in seed development were found. Nevertheless, they could be good candidates to investigate further since their function and descriptors coincide with the ones of the major sucrose transporters involved in the sucrose uptake during grain filling (*HvSUT1* and *HvSUT2*) that have been identified for barley (Weschke *et al.*, 2000; Sivitz *et al.*, 2005).

More robust evidences were found for the region on chromosome 3H. In this case the candidate gene identified was predicted to encode a 14-3-3 protein. These proteins are mainly involved in the regulation of the activity of starch biosynthesis enzymes and accumulation of starch granules that will determine the grain filling rate and so grain size and weight (Sehnke *et al.*, 2001; Zhang *et al.*, 2014). In fact, they have been shown to directly regulate this process in rice (Zhang *et al.*, 2014), wheat (Yu *et al.*, 2016), and barley (Alexander and Morris, 2006). Therefore, they could be considered as having a direct role on the establishment of the grain sink after anthesis and useful for improving grain filling in cereals.

These results suggest that the wild barley genome could contribute positively to the improvement of the crop performance by enhancing the ability to remobilise solutes into the grain. This hypothesis was hinted at in previous studies, suggesting genotypic differences in the

translocation and accumulation of carbohydrates and osmolytes in the RCSLs (Mendez *et al.*, 2011; del Pozo *et al.*, 2012), however further investigations using NILs for the targeted regions for QTL fine mapping would be necessary to confirm this assumptions.

To conclude, this study has identified 161 putative QTLs for important agronomic and developmental traits for some of which the wild barley genome has been found to contribute favourable alleles. Using the recently published genome sequence and gene annotation, candidates for important loci have been identified. Further population development using rounds of backcrossing would provide the means to fine map target chromosome regions and ultimately identify and clone the causative genes underlying target QTLs.

3.5 Conclusion

Wild barley germplasm constitutes a valuable genetic reservoir for broadening the genetic basis of the crop and achieving further agronomic and adaptive improvements through breeding programmes. However, because of the generalised negative effect of exotic genomes on the performance of modern varieties, optimised approaches are required to bring improvements into the elite barley gene-pools.

In the present study, a small set of 28 preselected RCSLs representing the entire genome of a wild donor accession have been successfully used in a QTL mapping study. An effective association analysis was designed to integrate the phenotypic information gathered for relevant agronomic and developmental traits over two growing season field trials (Chapter 2) and the high-throughput genotypic characterisation of the lines for over 1,800 polymorphic SNP markers from the Infinium iSelect 9K SNP chip platform. The methodology implemented in this study was not only useful to verify the effect of some known loci but also to locate new major QTLs with potential for further investigation and crop improvement. Therefore, this permanent genetic resource and the method implemented in this chapter could be applied in other studies aiming to explore the genetic variation in the wild barley genepool from the Fertile Crescent in the background of an adapted cultivar. Moreover, this group of lines offer the opportunity of conducting precise cost-effective QTL mapping studies for traits that would be difficult to assess in the larger population.

In this chapter, superior exotic QTLs have been identified that could enrich the genetic basis of cultivated barley. In particular, Caesarea 26-24 genome was found to potentially contribute genetic gains on yield by increasing the sink strength of the crop in the post-anthesis phase. New stable QTLs controlling grain weight and size for which interesting candidate genes associated with the transport of assimilates and the biosynthesis of starch in developing grains were identified. Specific assays to characterise the grain content composition in this set of 28 RCSLs should be straightforward and would provide evidence to support the hypothesis that Caesarea 26-24 alleles at the loci favoured grain-filling rate during the post-anthesis period compared to cv. Harrington alleles.

In addition to this, the association analysis revealed other chromosome regions explaining large phenotypic variation of traits conferring adaptation to different environments such as heading date and plant height. Many of the major QTLs found had been previously identified, and the pleiotropic effects exerted on plant performance were confirmed with observations in other experimental populations. However, in regard to qualitative traits, an interesting newly described QTL associated with the waxy non-glaucous surface of the spikes was identified as part of the study. Developing near isogenic lines for target loci would be necessary to fine map the QTL and identify causative genes controlling known loci such as the *earliness per se* locus

eps7L on chromosome 7H or the newly described QTL associated with the glossy spike on chromosome 1H. Additionally, sequencing and comparative analysis with other wild barley accessions or genetic backgrounds would aid not only to understand the adaptive role of these loci in wild barley natural habitats but also to assess their potential for fine-tuning flowering or enhancing drought tolerance in breeding programmes for specifically adapted lines.

Summing up, the information gathered in this study constitutes a first step towards map-based cloning of QTLs that could bring genetic gains to the crop and enhance adaptability to specific environments and also the methodology could be applied to QTL mapping in high-throughput phenotyping experiments.

4 Phenotyping the root system architecture of a subset of the RCSL population

Abstract

Exploring the genotypic variation for root system architectural traits and their function in water limited environments is essential for the development of more efficient crops. The aims of this study were to establish an experimental system to characterise seedlings root growth in controlled conditions and test whether the chromosome introgressions of the wild barley accession Caesarea 26-24 into cv. Harrington influenced root system characteristics and their development from early growth stages in the RCSLs. A two-dimensional paper growth pouch experiment and a trial using rhizotubes filled with field soil were used to evaluate the segregation for root traits in groups of selected RCSLs with sensitive and tolerant response to water deficit in the field.

Both experiments revealed genotypic variation for root traits that could potentially increase root system exploration to access soil moisture in water limited environments. Seminal root elongation rate, root diameter and gravitropic rate were found to diverge in groups of contrasting RCSLs. Despite this, these differences could not be associated with better performance under drought; they appear to coincide with improved root vigour and greater yield potential. The rhizotubes trial acted to confirm the genotypic variation in the root system development using two contrasting RCSLs and cv. Harrington. Genotypes with increased root growth in the pouches appeared to have an increased root length density when grown in field soil. However, the results obtained for root diameter were not conclusive across experiments and the variation in root angle or gravitropic response could not be validated using this method. Nevertheless, genotypic variation for important root traits was detected although a larger number of replicates would be recommended to investigate the effect of the water treatment on the RCSLs root system development.

The results obtained in the present study constitute the first attempt to quantify the RCSLs genotypic variation for root system architectural traits. It confirmed some previous results suggesting segregation for traits facilitating access to deep soil moisture and also the variation for root traits associated with the vigour of the plant. This variation is worthy of further investigations using a larger group of genotypes since it would aid the identification of major QTLs associated with specific root developmental traits.

4.1 Introduction

Selection for high yielding cultivars through traditional breeding has indirectly induced changes in the size and architecture of root systems of modern varieties compared to their wild ancestors or locally adapted varieties (Forster *et al.*, 2004). In barley, the root system size seems to have increased from wild barley to landraces to modern cultivars through the domestication process (Chloupek *et al.*, 2006). However, elite varieties are generally better suited to high-input agricultural systems that rely on heavy supply of nutrients and water and their performance under reduced fertilisation or limited irrigation is often not optimal (Yahiaoui *et al.*, 2014). Therefore, the optimisation of root system architecture through direct selection of root traits with potential to enhance water and nutrient uptake efficiently has become a new target for the development of crops able to maintain yields in low input agricultural systems (de Dorlodot *et al.*, 2007; Lynch, 2007).

Genotypic variation for important root traits in the three barley gene pools (wild, landraces and cultivars) from early stages of development could aid to improve abiotic stress tolerance in specific environments (Grando and Ceccarelli, 1995; Bengough *et al.*, 2004; Forster *et al.*, 2004). Wide crosses have shown the positive contribution of wild barley to enhance the performance of root architectural traits conferring adaptation to drought-prone environments (Naz *et al.*, 2012; Arifuzzaman *et al.*, 2014), and chromosome regions controlling traits such as root length and root volume have been identified using different genetic backgrounds (Chen *et al.*, 2010; Arifuzzaman *et al.*, 2014; Naz *et al.*, 2014; Robinson *et al.*, 2016). However, little is known about the benefits that this variation could bring to barley crop performance in stressed environments compared to other cereal crops. For example, favouring the deep rooting phenotype in rice has been found to mitigate the effect of water deficit and maintain high yield values under drought (Uga *et al.*, 2013). Similar observations have been found in wheat (Manschadi *et al.*, 2006).

The differential response to water deficit in the RCSLs (Chapter 2) and the evidence from previous work suggests segregation of root traits facilitates access to deep soil moisture during the grain filling period in this population (del Pozo *et al.*, 2012) and this observation motivated the present study. Here, a two-dimensional phenotyping approach was used to characterise the root growth parameters in groups of contrasting genotypes in their response to water deficit in the field. In addition, a rhizotube trial was conducted to determine the effect of water deficit on root system phenotype during seedling establishment. The objectives of the study were to i) establish a simple experimental system suitable for screening large numbers of barley plants at the seedling stage, ii) extract growth parameters for seminal and lateral roots from a time-lapse

dataset, iii) assess the effectiveness of the method for selecting lines contributing genetic variability for breeding programmes, and iv) find the effect of water scarcity on the responsiveness of the RCSLs root system.

4.2 Material and methods

4.2.1 Plant material

Recombinant Chromosomes Substitution Lines (RCSLs) with contrasting performance in field trials and cv. Harrington were used for investigating the root system development under controlled conditions. The selection was based on the drought tolerance index (DTI) or fraction of yield conserved in water stressed plants during the 2013 growing season in relation to the well-watered condition (see section 2.3.2.4); the field data obtained in 2014 was not available at the time of design of this experiment. This approach allowed lines with tolerant, sensitive and intermediate response to water stress to be defined based on one year of field data. The tolerant lines (OSU044 and OSU048) were low yielding genotypes with stable performance across water treatments showing less than 20% reduction in yield because of the stress (Table 4.1). In contrast, intermediate (OSU060 and cv. Harrington) and sensitive (OSU052 and OSU144) lines had high yield potential and the impact of drought was more marked, being around 25% reduction in the intermediate genotypes and up to 37% reduction in the sensitive lines (Table 4.1). Two methods were used to assess root architectural traits; pouches and rhizotubes with five RCSLs (OSU044, OSU048, OSU060, OSU052 and OSU144) and two RCSLs (OSU048 and OSU144) and cv. Harrington, respectively.

Table 4.1. Genotypes selected for root phenotyping in pouches and rhizotubes (*)

Genotype	Stable/Tolerant		Intermediate		Sensitive	
	OSU048*	OSU044	Harrington*	OSU060	OSU052	OSU144*
DTI 2013	1.16	1.11	1.03	1.03	0.86	0.87
Yield reduction (%)	15.2	19.2	24.7	24.8	37.5	36.9

4.2.2 Seed sterilisation and pre-germination

Prior to the start of the experiments, seeds of uniform size were surface sterilized by a vapour-phase sterilization method (Clough and Bent, 1998) using 100 ml sodium hypochlorite 4.5% and 5 ml concentrated HCl. The seeds were placed in open 50 ml Falcon tubes (Sarstedt AG & Co., Germany) and treated for an hour with chlorine fumes inside a desiccator jar placed in a fume hood. In addition, both experiments were conducted using pre-germinated seeds. For that sterilized seeds were sown on 10×10 cm germination paper (Anchor Paper, St. Paul, MN, USA) moistened with sterile distilled water, placed in Petri dishes and maintained vertically in a Qualicool™ cooled incubator at 20^{o}C with no light for 3 days.

4.2.3 2D pouch experiment

4.2.3.1 Experimental setup and growth conditions

Plants were grown in a controlled environment using a 2D pouch and wick system (Liao *et al.*, 2001; Hund *et al.*, 2009b) designed to monitor the root growth of barley seedlings over two weeks. Three-day-old seedlings were transferred to pouches that consisted of large sheets of germination paper (29.7 × 52 cm) pre-soaked in nutrient solution described below and held between an A3 size clear-Perspex plate and a 240 microns thick acetate sheet. Each germinated seed was placed in a slit at the top of the germination paper and attached to the plate with a drop of diluted Solvite wallpaper paste (Henkel Limited, Winsford, Cheshire, UK). Two foldback clips were attached to the sides of the plate and a clip hanger was used to hold the top of the plate. Each plate was wrapped in an aluminium foil to protect the roots from light and suspended into plastic boxes (60 cm × 68 cm × 46.5 cm) containing 30 L of nutrient solution in which approximately 10 cm of the germination paper was submerged. The nutrient solution was constantly aerated with a pneumatic pump in order to avoid precipitation of solutes (Fig, 4.1).

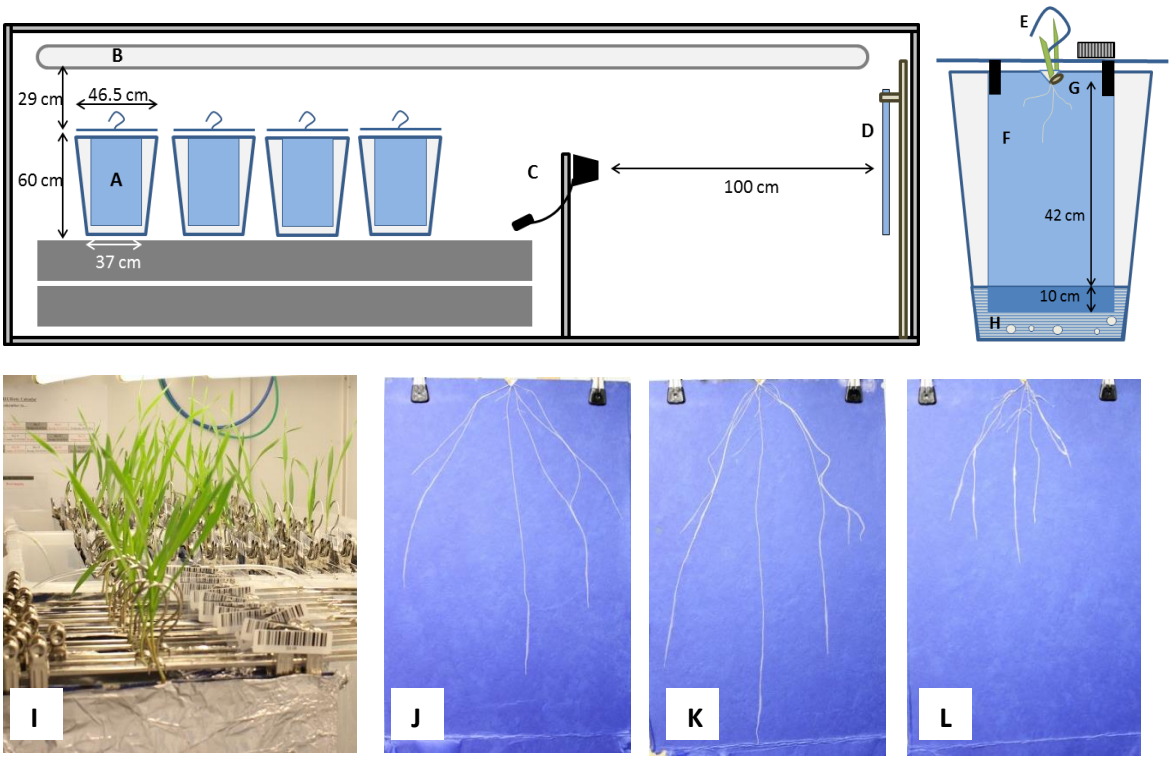

Figure 4.1 Pouch-and-wick experimental layout (A-H) Diagram of the experimental set up: (A) Each bucket contained two experimental replicates (16 seedlings, one per plate). (B) Fluorescent light. (C) Camera Canon EOS 550D on a tripod with a remote switch attached. (D) Easel with a plate. (E) Clip hanger with barcode. (F) A3 size clear-Perspex plate and acetate sheet with germination paper in between. Each plate was wrapped in foil. (G) Seedling attached in a slit on top of the germination paper. (H) Nutrient solution aeriated with a pneumatic pump. (I) Seedlings shoots after sixteen days of growth in pouches. (J-L) Three pouches last time-point images of plant roots in (J) cv. Harrington, (K) OSU144 and (L) OSU048.

The same nutrient solution was used to soak the germination paper and to fill in the plastic containers. The nutrient solution was prepared with deionized water and contained 300 mM NH_4Cl, 400 mM $Ca(NO_3)_2$, 400 mM KNO_3, 300 mM $MgSO_4$, 100 mM FeEDTA, 1 mM KH_2PO_4, 6 mM $MnCl_2$, 23 mM H_3BO_3, 0.6 mM $ZnCl_2$, 1.6 mM $CuSO_4$, 1 mM Na_2MoO_4, 1 mM $CoCl_2$. The nutrient solution was replaced every four days and its pH was adjusted to 5.5 using NaOH.

Eight replicates of each genotype were distributed in four plastic boxes, two complete replicates per box. Plants were grown for 15 days in a growth room under a 16/8 h day/night cycle at a constant temperature of 15^{o}C and 60% relative humidity approximately. Average light intensity during the day hours was 80 μmol quanta m^{-2} s^{-1} at plant height.

4.2.3.2 Phenotyping pipeline

i. Image acquisition

Pictures of each plate were taken every two days from day 2 to day 16 of the experiment with a Canon EOS 550D camera fixed on a tripod at a distance of 1 metre from the germination paper. Pictures were taken at exposure time 1/40 seconds, F-stop or aperture set of f/4 and 400 ISO. The plate was hung in an easel with a 1 m working distance. The aluminium foil and the acetate sheet were removed for taking the pictures and, before putting them back, the germination paper was sprayed with approximately 1 ml of the nutritive solution to ensure a homogeneous diffusion of the nutrients in the root system growing media.

ii. Harvest

After the last image, 18 day-old seedlings were removed from the plates. Shoots were excised from the roots and fresh weight of the shoots was recorded. Roots were detached from the germination paper and stored at room temperature in 50% ethanol. Roots were scanned (400 dpi) using an Epson Expression 10000XL professional DIN A3 scanner (Seiko Epson Corporation, Japan). Final root length and average root diameter were determined using WinRHIZO (Regent Instruments, Quebec, Canada). Shoots and roots were dried at 60^{o}C for 72 hours for determining the dry weight (DW) and the root to shoot ratio.

iii. Image processing

Image data was analysed by manual tracing of individual root trajectory using a Iiyama ProLite T2735MSC touch screen and using the Fiji software (Schindelin *et al.*, 2012). Raw images were first transformed into 8-bit grayscale images. For each genotype, the elongation rate of seminal and lateral roots as well as the branching rate of seminals were analysed for two time-steps, from day 2 to day 10 and from day 10 to day 16 of growth. Tracing was obtained using the freehand tool because many lateral roots were too small to be analysed efficiently using automated software such as Smartroot (Lobet, Pagès and Draye, 2011). ROI ("region of interest") formatted files produced for seminal and lateral roots of all the replicates for each genotype were then processed by a custom made macro so that the pixel coordinates of all roots in the images were exported as text files. Direct estimation of growth parameters for each genotype over these two growth intervals used formula derived by Hackett and Rose (1972):

Equation 6

$$e^{(0)} = \frac{dl^{(0)}}{n^{(0)}dt}$$

$$b^{(0)} = \frac{dn^{(1)}}{dt - T}$$

$$e^{(1)} = \frac{2dl^{(1)}}{n^{(0)}b^{(0)}(dt - T)^2}$$

here $e^{(0)}$ and $e^{(1)}$ (cm d^{-1}) are the elongation rate for respectively the seminal and lateral roots and $b^{(0)}$ (d^{-1}) is the branching rate of lateral roots. The parameter *dt* indicates the duration of the examined growth interval of 8 days (day 2 to day 10) and 6 days (day 10 to day 16) respectively, while $dl^{(0)}$ (cm) and $dl^{(1)}$ (cm) show the change in total seminal and lateral root length over the corresponding growth interval *dt.* The number of seminals is denoted by $n^{(0)}$, laterals is denoted by $n^{(1)}$ and any difference in the number of seminals over a time interval is given by $dn^{(0)}$. Since the number of seminal roots for the replicates of each genotype increased with time, $n^{(0)}$ was taken as the mean number of seminal roots during a given time interval where growth parameters were determined. For lateral roots, there was a time delay between the emergence of seminal and the emergence of lateral roots. The parameter *T (d)* is therefore the time it takes for lateral roots to emerge from the primary root during the time interval. In this experiment, it applied only to the first time-step (day 2 to day 8), since after 8 days, laterals had

emerged from all primary roots and was evaluated as the mean value of the time delay observed among the replicates of a single genotype.

Gravitropic rate or change in root angle was determined using Fiji software (Schindelin *et al.*, 2012) for stacked images from day 2 and day 4. In this case the images were first registered using the plugin Align Image by line ROI (Schindelin *et al.*, 2012). Registration of images from day 2 and day 4 used a line ROI with both ends of the ROI corresponding to the top and bottom of the slit on the germination paper. Two types of angles were recorded for these images. First the angle (α) of the root at day 2 was measured using the Straight Line ROI. Then, the change in angle ($d\alpha$) taking place for the same root between day 2 and day 4 was recorded using Segmented Line ROI and angle measurement. Three randomly selected seminal roots of each plate were measured. The gravitropic rate parameter (g_0) is defined as the fraction of the decrease in angle per unit time and it was determined for each genotype using the information gathered for a total of 24 seminal roots as follows:

Equation 7

$$g^{(0)} = \frac{d\alpha}{\alpha dt}$$

where *dt* is equal to 2, since the change in angle is measured for an interval of 2 days.

4.2.3.3 Statistical analysis

Statistical analysis of the genotypic effects on root traits measured was performed using a two factorial mixed model considering the genotype, the time-step (day 2 to day 10, day 10 to day 16 of the experiment) and their interaction as fixed effects. The statistical significance for the fixed model effect was assessed by using a chi-squared Wald test. The experimental replicate was considered as the random effect. Genstat 17th Edition (VSN International, UK) was used for this analysis.

4.2.4 Rhizotubes trial

4.2.4.1 Experimental setup and growth conditions

Soil columns or rhizotubes were used to evaluate the effect of water deficit on early stage of seedling development in two contrasting RCSLs (OSU144 and OSU048) and cv. Harrington

(Fig. 4.2). Topsoil collected from the experimental trial site (see section 2.2.2) and sieved through a 10 mm mesh was used to fill the tubes. Preliminary evaluations of the soil physical characteristics (see section 2.2.2.3) using three samples (40 mm height, 55 mm diameter) of sieved field soil at 1.2 g/cm^3 dry bulk density aided the design of the experiment and planning of the water treatment since it allowed the estimation of the soil matric potential at field capacity and an approximated assessment of the water contained in the rhizotubes throughout the experiment.

The rhizotubes consisted of 100 cm length and 5 cm diameter open end PVC tubes with nylon gauze covering the base of the tube. First, the tubes were lined with a sheet of black polythene to enable the extraction of intact soil columns at the end of the experiment, then, the bottom of the tube was filled with gravel to facilitate drainage and the remaining 80 cm length soil column packed at 1.2 g/cm^3 bulk density was established on top. The packing was performed in eight layers 10 cm thick to achieve a uniform soil compaction along the column; in addition, the interface between soil layers was roughened prior to packing of next layer in order to reduce the shear stress between layers and allow a more continuous soil structure in the column. Finally, water was added to reach 80% field capacity and the soil column was left to settle for 2 days prior to transplanting pairs of five-day old seedlings. Seedlings were sown in the tubes the 25th April 2015. After two days of establishment, one seedling was kept per tube and a layer of fine grit was added on top of the soil surface in order to reduce water evaporation. After this period, two water treatments were established.

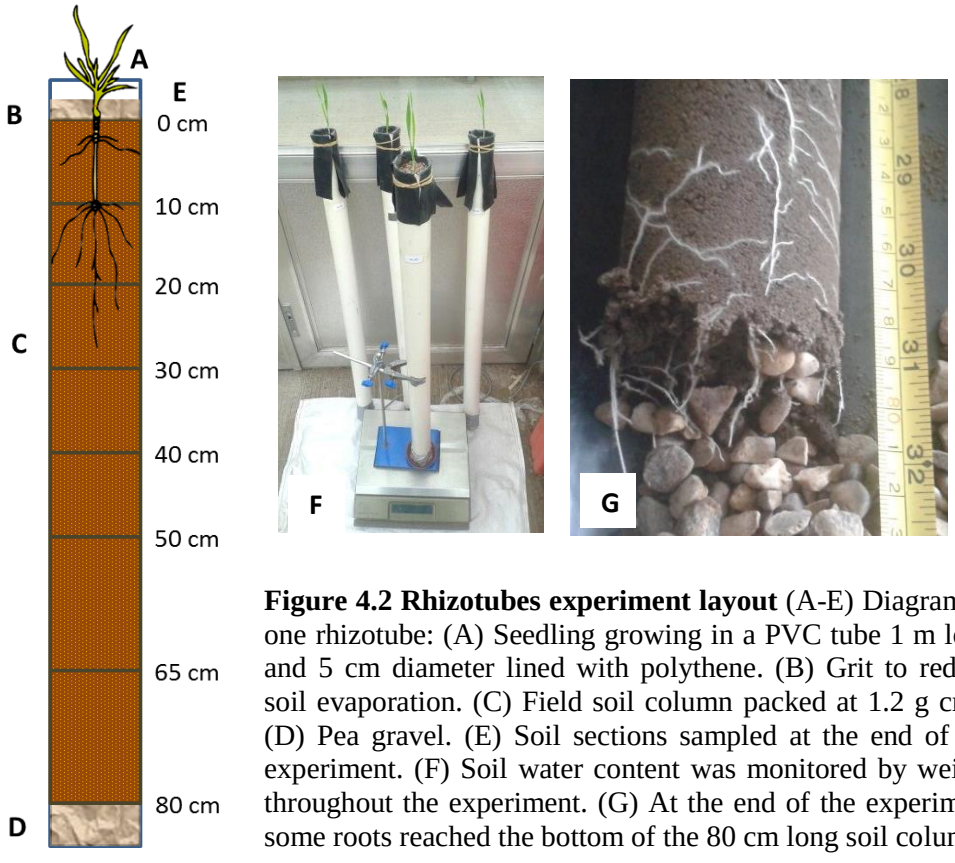

Figure 4.2 Rhizotubes experiment layout (A-E) Diagram of one rhizotube: (A) Seedling growing in a PVC tube 1 m long and 5 cm diameter lined with polythene. (B) Grit to reduce soil evaporation. (C) Field soil column packed at 1.2 g cm^{-3}. (D) Pea gravel. (E) Soil sections sampled at the end of the experiment. (F) Soil water content was monitored by weight throughout the experiment. (G) At the end of the experiment some roots reached the bottom of the 80 cm long soil column.

Control plants were kept at 80% field capacity all through the experiment by weighing the rhizotubes and adjusting the water content every second day with distilled water. In contrast, no irrigation was applied to the water stress tubes after seedling establishment. Three replicates (18 rhizotubes in total) completely randomised were grown for 25 days in the glasshouse under approximately 16/8 h day/night cycle maintained with supplemental lighting allowing a minimum light intensity of 200 µmol quanta m^{-2} s^{-1}. Temperature was allowed to fluctuate between 15 and 25^{o}C.

4.2.4.2 Phenotypic evaluation

i. Shoot phenotype

Phenotypic data was obtained on the last day of the experiment. In order to determine any reduction in vegetative growth as a consequence of the lack of water, the height of the newest leaf auricle on the main stem and the number of leaves developed were recorded. In addition, leaf area was measured with a LI-3000A portable area meter (LI-COR Biosciences, USA) on the fourth unfolded leaf of each seedling after which a 7 cm length leaf sample from the tip was taken to estimate the relative water content (RWC) reached at the end of the experiment according to Barr and Weatherley (1962) as follows:

Equation 8

$$RWC(\%) = [W_0 - W_D / W_T - W_D] \times 100$$

where W_0 is the leaf sample fresh weight, W_D is the dry weight after 48 hours at 70^{o}C and W_T is the sample turgid weight after hydration of the fresh samples in closed Falcon tubes with 15 ml de-ionized water for 3 hours at room temperature.

ii. Roots phenotype

The 80 cm long soil column was divided into seven sections from the top (five 10 cm long sections from 0 to 50 cm depth and two 15 cm long sections from 50 to 80 cm depth) to estimate the root distribution and water content in the soil column profile. Soil samples were taken to estimate the water content of each soil column section. The roots were gently hand washed with water to remove the soil and stored at room temperature in 50% ethanol until they

were scanned (400 dpi) using an Epson Expression 10000XL professional DIN A3 scanner (Seiko Epson Corporation, Japan). Root length per soil volume (cm cm^{-3}), average root diameter (mm), root volume (cm^3) and root surface area (cm^2) were determined from image analysis of scanned washed roots of each soil section using WinRHIZO (Regent Instruments, Quebec, Canada). Finally, root to shoot ratio and root dry matter density (mg cm^{-3}) were determined from samples dried at 60^{o}C for 72 hours.

4.2.4.3 Statistical analysis

Each trait was analysed using a mixed model approach to test the effect of the genotype, the treatment, the soil section (when required) and their interaction. The chi-squared based Wald-test was used to evaluate the statistical significance for the fixed term of the model. Genstat 17th Edition (VSN International, UK) was used for this analysis.

4.3 Results

4.3.1 2D- pouch experiment

Two groups of data were generated in this experiment:

i) Root growth parameters characterising the development of seminal and lateral roots at two time-steps throughout the experiment: seminal and lateral roots elongation rate and number, branching rate and seminal roots variation in root angle were obtained from the images.

ii) Last time point data giving an overall picture of the root system phenotype at the end of the experiment, when seedlings showed 2 or 3 unfolded leaves (GS12–GS13): total root length and average root diameter obtained from roots scans.

i. RCSLs root growth parameters

Seminal roots

The RCSLs developed approximately seven seminal roots during the first days of seedling growth (day 2 to day 10) when the elongation rate of seminal roots was intensified compared to the second half of the experiment (day 10 to day 16), with an average of 1.96 cm and 1.63 cm per day respectively. OSU044 appears to have slightly fewer seminal roots (5.88 on average) compared to the other genotypes (cv. Harrington: 7.38, OSU048: 6.94, OSU060: 7.25, cv. Harrington: 7.38, OSU052: 7.13, OSU144: 7.13 on average) although no genotypic differences were found for this trait when analysed at the end of the experiment. The seminal root elongation rate was the most discriminating variable across the RCSLs ($P<0.001$, Table 4.2). For example, OSU048 (stable but limited performance) had a very low and uniform elongation rate all through the experiment (0.94±0.04 cm/day and 1.13±0.14 cm/day from day 2 to 10 and day 10 to 16 respectively). In contrast, OSU144 (sensitive but high yield potential) showed an overall increase in elongation rate for the seminal roots, with a greater elongation rate from day 2 to 10 (2.8±0.2 cm/day) than from day 10 to 16 (2.3±0.1 cm/day). This trend was observed for all the genotypes except the two tolerant and stable lines (OSU048 and OSU044) (Fig. 4.3).

Lateral roots

Branching and lateral root elongation rates showed large variation at the genotype level due to the stochasticity of this growth parameter. For all genotypes, the number of lateral roots emerged from day 2 to day 10 of the experiment was greater than the number of lateral roots that emerged from day 10 to day 16. Genotypic differences were also found for lateral roots

elongation rate (P<0.05, Table 4.2). Lateral roots of OSU048 grew vigorously from day 10 to 16 (0.7±0.3 cm/day) and this resulted in a much larger total lateral root length at the end of the experiment (40.2±7.0 cm), compared to genotypes such as OSU044 (4.5±1.4 cm) and OSU052 (13.3±3.5 cm) which had a lateral root growth rates significantly lower (Fig. 4.3).

Root gravitropic rate

Variation in the root angle of seminal roots across genotypes was detected. In this case the change in root growth angle was measured from day 2 to day 4 of the experiment in three randomly selected seminal roots per seedling. In cv. Harrington and OSU052 the change in root angle was less than in OSU044 and OSU060 which seem to have a narrower spread of roots (Fig. 4.3). However, direct comparison of this result with the images obtained for each genotype indicate that this measure may be of limited value because it was obtained at a fixed point in time and it is not representative of the overall plant behaviour. Genotypes OSU060 and cv. Harrington were visually very similar, exhibiting a wider spread of root, compared to genotypes such as OSU052 and OSU144 where gravitropic rate looked more accentuated (Appendix 19).

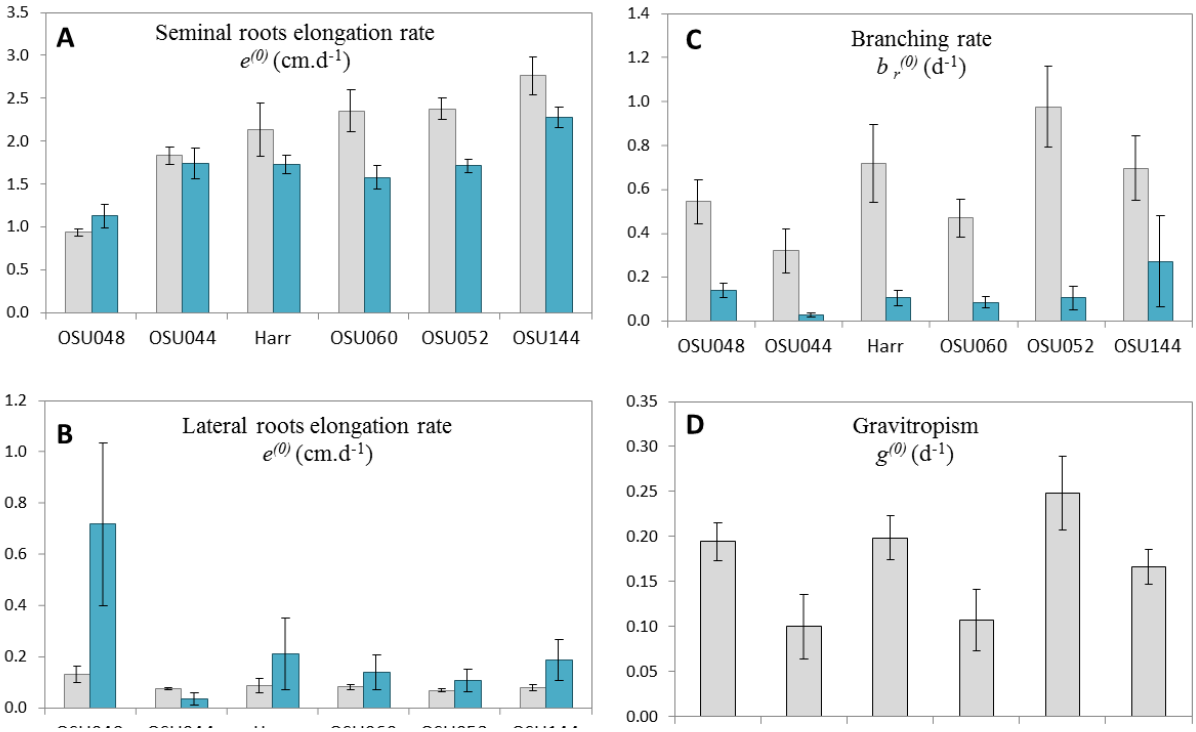

Figure 4.3 Variations in root growth parameters with time and as a function of genotype. Bar charts represent mean values (+/- SE) for (A) seminal roots elongation rate (cm.d^{-1}), (B) lateral roots elongation rate (cm.d^{-1}) (C) branching rate (number of branches (root)/day). Growth parameters from Day 2 to Day 10 are plotted with grey shading, and growth parameters from Day 10 to Day 16 are plotted with blue shading. (D) Genotypes mean value for gravitropic rate measured from Day 2 to Day 4. Error bars represent standard error of the mean

Table 4.2 Root growth parameters mean values (±SE) for 5 RCSLs and cv. Harrington evaluated in a 2D pouch experiment. Genotype and time-step overall means as well as the combination genotype by time-step means are indicated. Seminal and lateral roots number, elongation rate and branching rate were calculated for day 2 to day 10 and day 10 to day 16. Gravitropic rate was measured as the change in seminal angle from day 2 to day 4.

Trait/time-step	OSU048	OSU044	Harrington	OSU060	OSU052	OSU144	Time-step mean	Effect[2]
Seminal root number								
day2–day10	6.88±0.30	5.75±0.25	7.25±0.48	7.25±0.25	7.00±0.27	7.00±0.41	6.88±0.15	G^{ns}
day10–day 16	7.00±0.33	6.00±0.41	7.50±0.50	7.25±0.25	7.25±0.25	7.25±0.48	7.06±0.16	$T\text{-}s^{ns}$
Genotype mean	*6.94±0.21*	*5.88±0.23*	*7.38±0.32*	*7.25±0.16*	*7.13±0.18*	*7.13±0.30*		$GxT\text{-}s^{ns}$
Seminal elongation rate ($cm.d^{-1}$)								
day2–day10	0.94±0.04	1.83±0.10	2.13±0.31	2.35±0.25	2.38±0.13	2.76±0.22	1.96±0.13	G^{***}
day10–day 16	1.13±0.14	1.74±0.18	1.73±0.11	1.58±0.14	1.71±0.08	2.28±0.12	1.63±0.08	$T\text{-}s^{***}$
Genotype mean	*1.03±0.07*	*1.79±0.10*	*1.93±0.17*	*1.97±0.20*	*2.05±0.11*	*2.52±0.15*		$GxT\text{-}s^{***}$
Lateral Root number								
day2–day10	13.50±2.82	5.75±0.85	14.00±4.02	11.50±3.01	13.12±2.50	10.25±1.32	11.84±1.16	G^{ns}
day10–day 16	19.25±3.12	6.75±1.11	18.50±3.59	15.25±3.09	17.25±3.30	22.25±10.65	16.97±1.85	$T\text{-}s^{***}$
Genotype mean	*16.38±2.16*	*6.25±0.68*	*16.25±2.64*	*13.38±2.12*	*15.19±2.07*	*16.25±5.46*		$GxT\text{-}s^{ns}$
Lateral elongation rate[1] ($cm.d^{-1}$)								
day2–day10	0.13±0.17	0.07±0.01	0.09±0.07	0.08±0.03	0.07±0.02	0.08±0.04	0.09±0.01	G^{*}
day10–day 16	0.72±0.03	0.03±0.00	0.21±0.03	0.14±0.01	0.11±0.01	0.19±0.01	0.28±0.09	$T\text{-}s^{ns}$
Genotype mean	*0.42±0.32*	*0.05±0.02*	*0.15±0.14*	*0.11±0.07*	*0.09±0.05*	*0.13±0.08*		$GxT\text{-}s^{**}$
Branching rate (d^{-1})								
day2–day10	0.54±0.10	0.32±0.10	0.72±0.18	0.47±0.09	0.98±0.19	0.70±0.15	0.65±0.07	G^{ns}
day10–day 16	0.14±0.03	0.03±0.01	0.10±0.04	0.08±0.03	0.10±0.05	0.27±0.21	0.12±0.03	$T\text{-}s^{**}$
Genotype mean	*0.34±0.07*	*0.17±0.07*	*0.41±0.14*	*0.28±0.08*	*0.54±0.15*	*0.48±0.14*		$GxT\text{-}s^{ns}$
Gravitropic rate (d^{-1})	0.19±0.02	0.10±0.04	0.20±0.02	0.11±0.03	0.25±0.04	0.17±0.02		

1 The stats analysis was carried out on the logarithm of the trait

2 Analysis of the genotype (G) and time- step (T-s) and their interaction (GxT-s) effect on root parameters using a mixed effect model. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

ii. Last time point root phenotype

Large differences (P<0.001) in the root system phenotype across the RCSLs were observed at the end of the experiment, presumably as a consequence of the genotypic differences in root growth parameters throughout the experiment. For example, the genotypes with the lowest seminal root elongation rate (OSU048 and OSU044) produced the shortest root system (116.7±7.9 cm and 136.8±7.0 cm in average) contrasting with OSU052 and OSU144 which had the largest seminal root elongation rate throughout the experiment and the longest root system (corresponding to 176.2±3.8 cm and 191.0±7.4 cm) (Fig. 4.4). Interestingly, genotypes with longer root system tended to show thinner roots and the opposite (Fig. 4.5). Additionally, the allocation of dry matter in shoots and roots differed between genotypes (Fig. 4.4). Root to shoot ratio in OSU048 (56.1%) was significantly larger compared to the other RCSLs, with OSU060 having the smallest value (34.7%). Finally, OSU060 had similar root growth parameters and root system phenotype to cv. Harrington, suggesting that the small exotic introgressions present in this line had a negligible effect on the root system at early stage of development (Fig. 4.3).

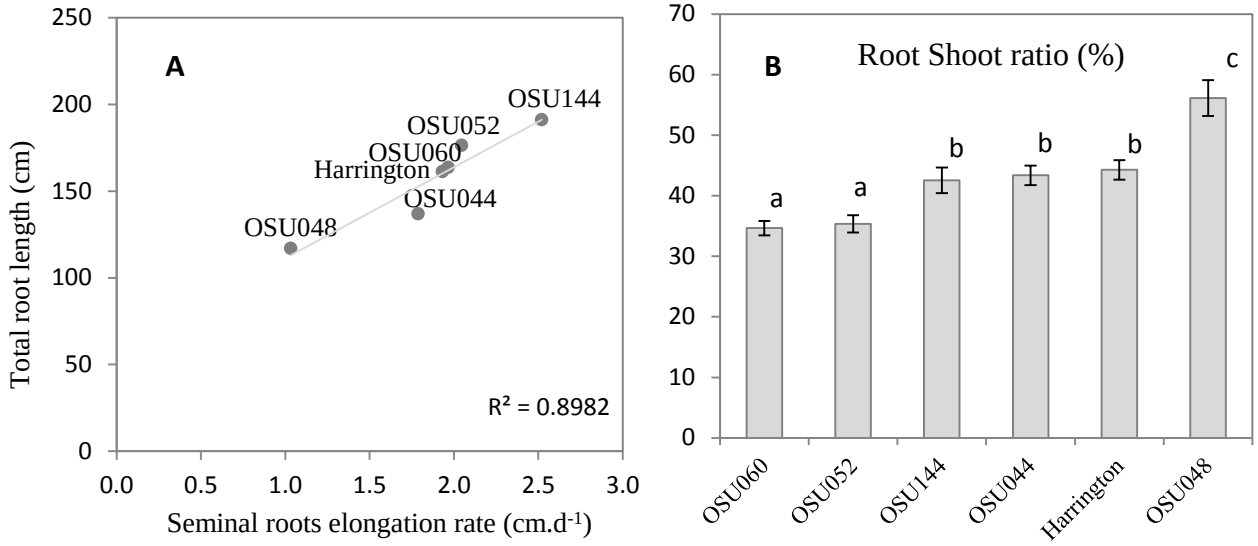

Figure 4.4. Root and shoot growth measured in eighteen-day old seedlings. (A) Relationship between mean seminal elongation rate throughout the experiment for each genotype and total root length measured in scanned roots. **(B)** Average root to shoot ratio (%) for each genotype. Different letters indicate significant differences (*P*<0.001, Fisher's LSD test). Error bars correspond to the standard error of the mean.

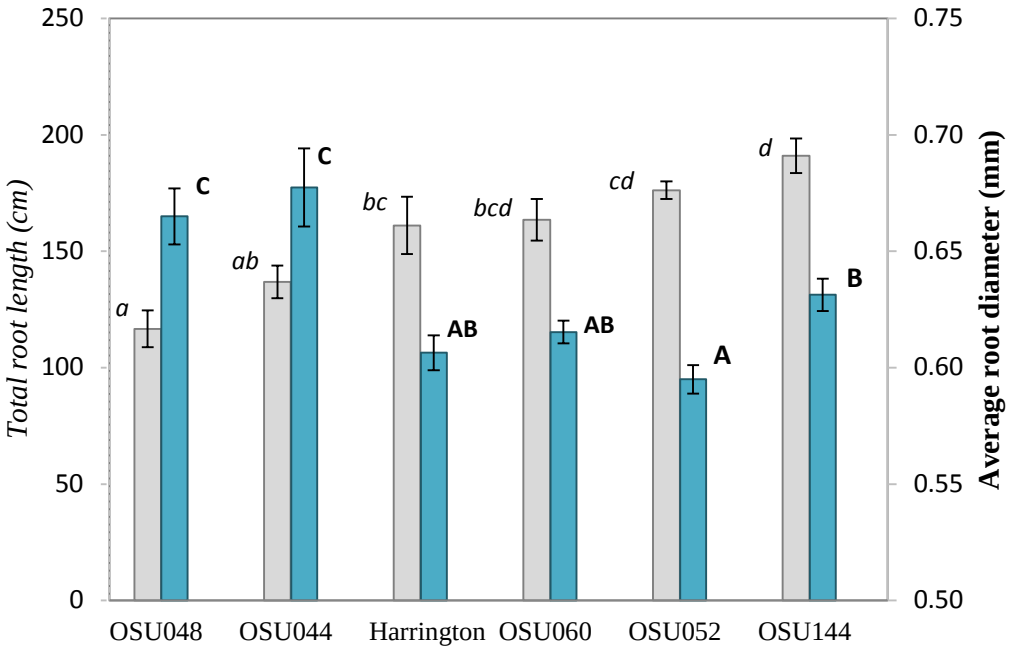

Figure 4.5. Root system characterisation after sixteen days of growth in pouches. Mean total root length (grey bars, italics letters) and average root diameter (blue bars, bold letters) for each genotype measured in washed roots with WinRHIZO. Different letters indicate significant differences (P<0.001, Fisher's LSD test). Error bars indicate standard error of the mean.

4.3.2 Rhizotubes experiment

The rhizotubes experiment was designed to assess the RCSLs root system development response to limited water. Two RCSLs (OSU048 and OSU144) with contrasting root system size and seminal elongation rate were selected for a preliminary evaluation.

After 25 days of growth in soil columns, plants had reached growth stage GS16 – GS17, i.e. seedlings showed six or seven unfolded leaves (Zadoks *et al.*, 1974). In some cases, signs of tillering initiation were observed. A progressive decrease in soil column water content was registered throughout the experiment in both treatments (Fig. 4.6), being more acute in the droughted plants and leading to greater differences in the soil moisture profile between treatments at the end of the experiment (Fig. 4.6). Soil water content in droughted rhizotubes was small and consistent across the soil column, maintained at around 0.12 cm^3 cm^{-3} from top to bottom. In contrast, the control rhizotubes which were kept at 80% field capacity throughout the experiment, showed a water content gradient in the soil column varying from 0.23 cm^3 cm^{-3} at the top (0 to 10 cm) to 0.14 cm^3 cm^{-3} at the bottom layer (65 to 80 cm).

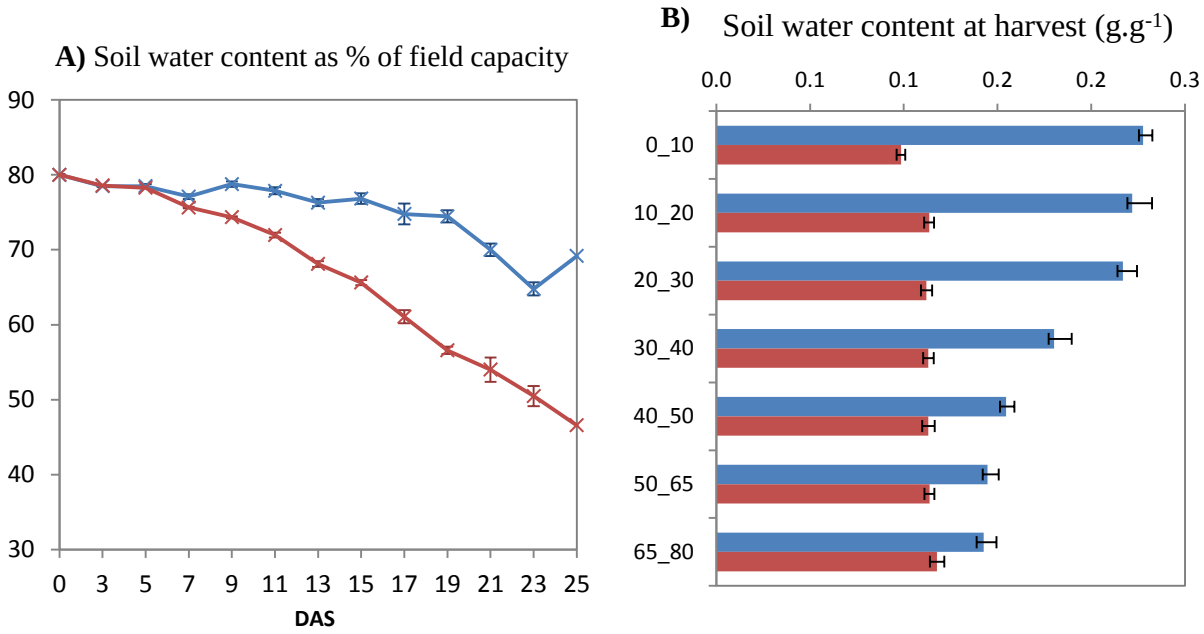

Figure 4.6 Rhizotubes water content. **(A)** Estimated soil water content (%) in the soil column (y axes) from day 0 to day 25 of seedling growth (X axes) in rhizotubes for the control (blue) and drought treatment (red). **(B)** Water content in the droughted (red) and irrigated (blue) soil columns at the time of harvest for seven different soil sections (y axes). Error bars indicate standard error of the mean.

4.3.2.1 Drought effect on seedlings development

Water deficit affected aboveground seedling development significantly (Table 4.3). After 25 days of growth with no irrigation, droughted seedlings were shorter (7.2 cm auricle height) and developed fewer leaves (6.4 leaves) in relation to the control plants (9.5 cm height and 8.6 leaves in average). Although no visual differences in leaf appearance between treatments were observed, the leaf relative water content was considerably less under drought (83.5%) than in irrigation (94.3%). Also, no differences in the distribution of root biomass in the soil profile or root morphology were detected between treatments. Nevertheless, readjustment in seedling growth depending on the water available was reflected in the significant ($P<0.001$) differential allocation of biomass or root to shoot ratio corresponding to 61% under stress and 41% under control, essentially due to a significant reduction in shoot biomass under drought.

Table 4.3 Shoot phenotypic characterisation for OSU048, cv. Harrington and OSU144 after 25 days of growth in rhizotubes in two water regimes (control and drought). Overall means for each genotype, treatment and genotype–treatment combination (±SE) are indicated.

Shoot trait Treatment	OSU048	Harrington	OSU144	Treatment mean	Effect[1]
No leaves					
control	8.00±1.53	10.00±1.00	7.67±1.45	8.56±0.77	G^{ns}
drought	7.00±0.58	7.33±0.67	5.00±0.00	6.44±0.44	T^{*}
Genotype mean	*7.50±0.76*	*8.67±0.80*	*6.33±0.88*		GxT^{ns}
Height auricle (cm)					
control	6.60±0.31	10.50±0.29	11.33±0.33	9.48±0.75	G^{***}
drought	6.17±0.44	6.10±0.74	9.33±0.33	7.20±0.60	T^{***}
Genotype mean	*6.38±0.26*	*8.30±1.05*	*10.33±0.49*		GxT^{**}
Leaf area (cm^2)					
control	15.69±4.32	17.65±2.75	21.00±1.42	18.12±1.72	G^{ns}
drought	16.57±2.70	17.52±1.82	18.03±2.01	17.37±1.13	T^{ns}
Genotype mean	*16.13±2.29*	*17.59±1.48*	*19.52±1.29*		GxT^{ns}
Leaf length (cm^2)					
control	23.43±4.10	25.67±0.97	27.93±1.17	25.68±1.42	G^{ns}
drought	25.23±2.80	25.00±0.23	26.77±1.84	25.67±1.01	T^{ns}
Genotype mean	*24.33±2.26*	*25.33±0.47*	*27.35±1.01*		GxT^{ns}
Leaf width (cm^2)					
control	0.67±0.09	0.67±0.09	0.73±0.03	0.69±0.04	G^{ns}
drought	0.67±0.03	0.70±0.06	0.67±0.03	0.68±0.02	T^{ns}
Genotype mean	*0.67±0.04*	*0.68±0.05*	*0.70±0.03*		GxT^{ns}
RWC (%)					
control	94.70±2.65	93.80±2.50	94.44±1.10	94.31±1.11	G^{ns}
drought	81.34±6.15	82.86±3.45	86.34±0.36	83.51±2.17	T^{**}
Genotype mean	*88.02±4.23*	*88.33±3.10*	*90.39±1.88*		GxT^{ns}
Shoot dry weight (g)					
control	0.29±0.09	0.43±0.04	0.43±0.04	0.38±0.04	G^{ns}
drought	0.28±0.02	0.27±0.03	0.28±0.01	0.28±0.01	T^{*}
Genotype mean	*0.29±0.04*	*0.35±0.04*	*0.36±0.04*		GxT^{ns}
Root to shoot ratio					
control	0.50±0.08	0.39±0.05	0.36±0.02	0.41±0.04	G^{ns}
drought	0.58±0.06	0.67±0.07	0.59±0.02	0.61±0.03	T^{***}
Genotype mean	*0.54±0.05*	*0.53±0.07*	*0.48±0.05*		GxT^{ns}

1 Analysis of the genotype (G) and time- step (T-s) and their interaction (GxT-s) effect on root parameters using a mixed effect model. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

Table 4.4 Root phenotypic characterisation for OSU048, cv. Harrington and OSU144 after 25 days of growth in rhizotubes in two water regimes (control and drought). Soil column overall means for each genotype, treatment and genotype–treatment combination (±SE) are indicated.

Root trait Treatment	OSU048	Harrington	OSU144	Treatment mean	Effect[1]
Average root diameter (mm)					
control	0.35±0.02	0.38±0.01	0.44±0.02	0.39±0.01	G^{***}
drought	0.37±0.01	0.37±0.01	0.47±0.01	0.40±0.01	T^{ns}
Genotype mean	*0.36±0.01*	*0.38±0.01*	*0.46±0.01*		GxT^{ns}
Root length density (cm.cm^{-3})					
control	1.74±0.19	2.28±0.11	1.80±0.11	1.94±0.09	G^{***}
drought	2.02±0.12	2.12±0.11	1.67±0.11	1.94±0.07	T^{ns}
Genotype mean	*1.88±0.11*	*2.20±0.08*	*1.74±0.08*		GxT^{ns}
Root surface area (cm^2)					
control	41.53±6.40	62.89±5.85	58.72±6.19	54.38±3.68	G^{**}
drought	53.67±4.69	55.76±4.11	55.28±4.36	54.90±2.50	T^{ns}
Genotype mean	*47.60±4.03*	*59.32±3.58*	*57.00±3.75*		GxT^{*}
Root volume (cm^3)					
control	0.38±0.09	0.63±0.08	0.69±0.10	0.57±0.05	G^{***}
drought	0.52±0.06	0.54±0.06	0.66±0.06	0.57±0.03	T^{ns}
Genotype mean	*0.45±0.05*	*0.59±0.05*	*0.68±0.06*		GxT^{ns}
Root dry matter density (mg.cm^{-3})					
control	0.09±0.01	0.11±0.01	0.10±0.01	0.10±0.01	G^{ns}
drought	0.11±0.01	0.11±0.00	0.11±0.00	0.11±0.00	T^{ns}
Genotype mean	*0.10±0.01*	*0.11±0.00*	*0.10±0.00*		GxT^{ns}

1 Analysis of the genotype (G) and time- step (T-s) and their interaction (GxT-s) effect on root parameters using a mixed effect model. Statistical values (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). ***P<0.001,**P<0.01,*P<0.05, ns: not significant

4.3.2.2 Differences between genotypes

Genotypes differed significantly in seedling height which was also affected by water treatment. OSU048 and OSU144 were the two extreme lines, with 6.6 cm and 11.3 cm average height of the newest auricle in the control treatment. The lack of water significantly reduced the height of OSU144 and cv. Harrington whereas OSU048 maintained similar values (Fig 4.7); cv. Harrington showed the largest reduction in height due to water deficit.

In regard to the root system, genotypic differences were detected for root length density and root diameter (P<0.001) with the subsequent effects on total root volume and root surface (P<0.01) per soil section (Table 4.4). For example, cv. Harrington showed greater root length per unit of soil volume (2.2 cm cm^{-3}) compared to the RCSLs (1.9 cm cm^{-3} OSU048 and 1.7 cm cm^{-3}

OSU144); however, OSU144 was significantly greater in average root diameter (0.46 mm) compared to the cv. Harrington (0.38 mm) and OSU048 (0.36 mm). The increased root length in cv. Harrington and larger root diameter in OSU144, improved total root volume (0.59 cm^3 and 0.68 cm^3 respectively) and therefore, total root surface area (59.3 cm^2 and 57.0 cm^2 respectively) per soil section of these two genotypes in relation to OSU048, which showed significantly diminished performance overall for the traits (0.45 cm^3 root volume and 47.6 cm^2 root surface area) (Table 4.4). The differences in the interaction with the treatment were generally not significant, probably due to the reduced number of experimental replicates; however, the root surface area in OSU048 increased notably ($P<0.05$) under drought conditions (from 41.3 cm^2 under irrigation to 53.7 cm^2 under drought) compared to the other genotypes which actually showed the opposite trend for the trait although the difference was not significant (Fig 4.7).

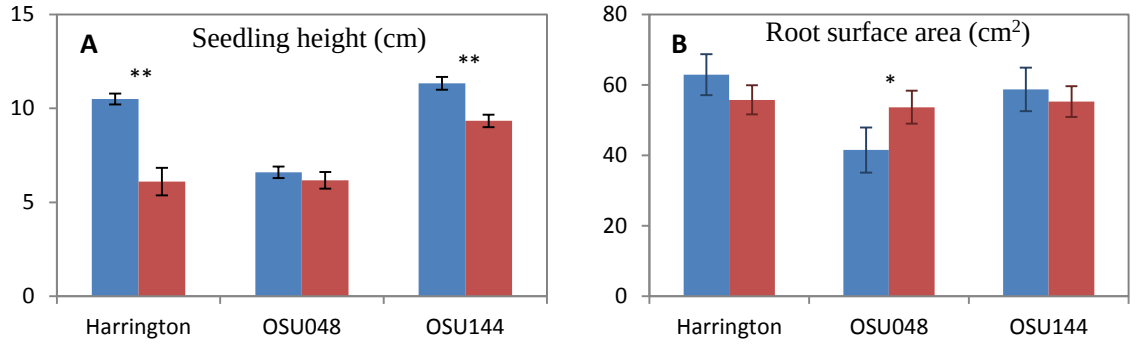

Figure 4.7 Genotypic variation in response to the water treatment for seedling height (A) and average root surface area (B) measured at the end of the experiment for the control (blue) and drought (red) treatment in the three genotypes evaluated. Error bars represent standard error of the mean and asterisk represent significant difference between water treatments for a genotype (**P<0.01, *P<0.05)

The root morphology and distribution varied significantly across soil sections (Table 4.5). Average root diameter increased gradually with depth, reaching the largest values in the deepest soil layer (0.5 mm). In addition, most of the roots developed in the middle-top layers, from 20 to 40 cm depth, where the root length per volume of soil was found augmented (2.3 cm cm^{-3}) compared to the top ten centimetres (1.2 cm cm^{-3}) or the bottom layers (1.8 cm cm^{-3}). No significant differences were detected in the distribution or morphology of roots with depth across genotypes or as a consequence of the water treatment, probably due to the reduced number of replicates. However different trends were found between genotypes, especially for OSU048. This genotype seems to increase the root surface area considerably in response to water stress in the middle (30 to 50 cm) and lower (50 to 80 cm) soil layers whereas OSU144 and cv. Harrington did not appear to adjust its root system development in response to water stress (Fig. 4.8).

Table 4.5. Root morphology and distribution in the soil column sections. Mean values (±SE) obtained for 18 rhizotubes

Soil column section (cm)	Av. diameter (mm)*	Root length density ($cm.cm^{-3}$)*	Root surface area (cm^2)*	Root volume (cm^3)*	Dry matter density ($mg.cm^{-3}$)
0-10	0.34±0.01	1.25±0.10	25.89±1.96	0.22±0.02	0.11±0.01
10-20	0.34±0.01	2.07±0.12	43.00±2.51	0.37±0.03	0.09±0.01
20-30	0.38±0.01	2.31±0.13	53.07±2.90	0.50±0.03	0.11±0.01
30-40	0.39±0.01	2.32±0.12	54.85±3.01	0.54±0.04	0.10±0.01
40-50	0.41±0.01	1.99±0.12	49.99±3.41	0.52±0.05	0.10±0.01
50-65	0.42±0.01	1.85±0.12	72.59±5.61	0.78±0.08	0.09±0.01
65-80	0.50±0.02	1.80±0.14	83.11±7.59	1.06±0.12	0.12±0.01

*Traits accounting for significant differences between soil sections P<0.001

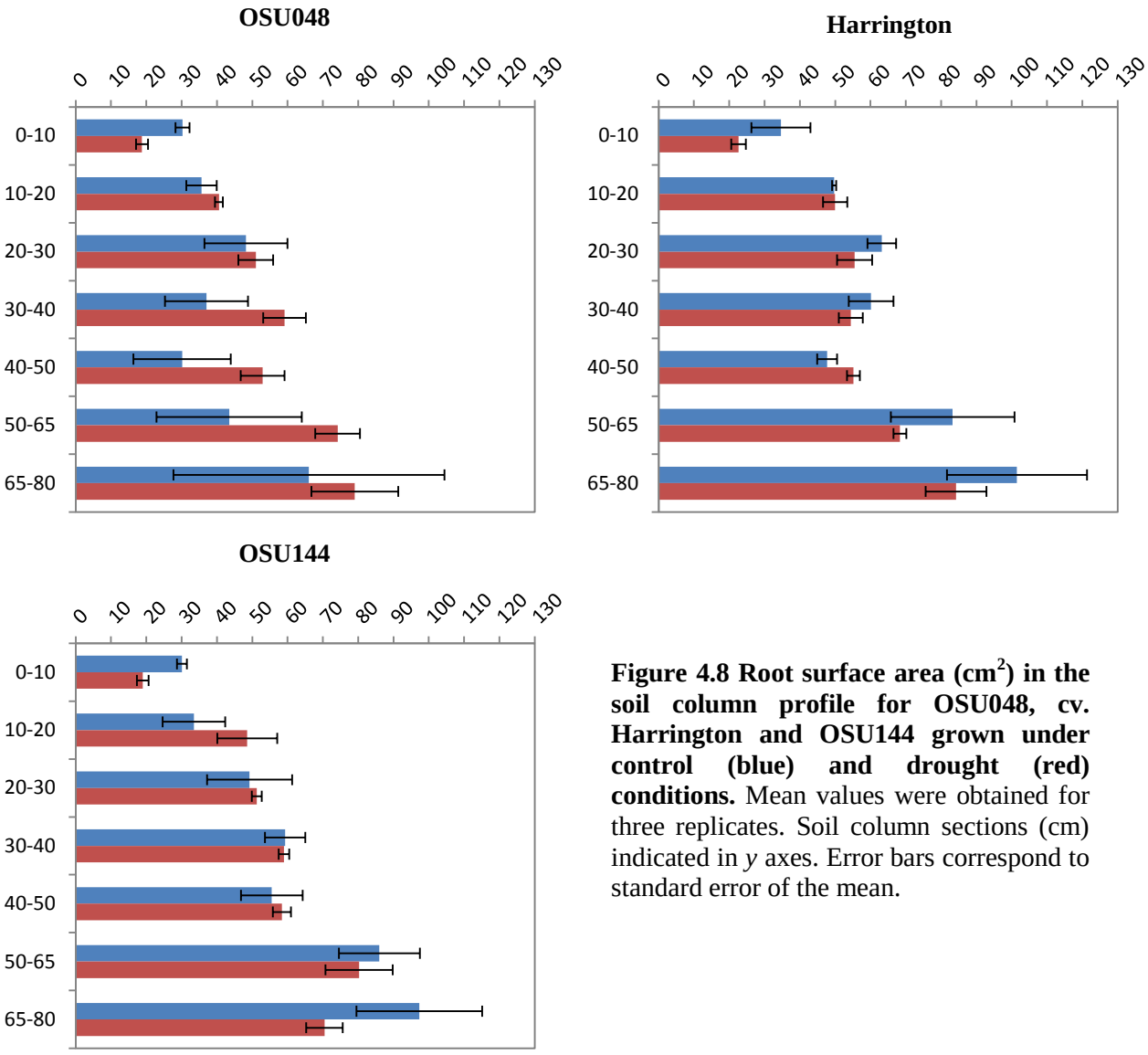

Figure 4.8 Root surface area (cm^2) in the soil column profile for OSU048, cv. Harrington and OSU144 grown under control (blue) and drought (red) conditions. Mean values were obtained for three replicates. Soil column sections (cm) indicated in *y* axes. Error bars correspond to standard error of the mean.

4.4 Discussion

The aim of this chapter was to investigate the effect of the wild barley chromosome introgressions in a subset of the RCSLs on root system development during the early stages of growth. To this end, two experiments under controlled conditions were conducted to assess different root traits that could influence the access to soil water resources, a 2D pouch and a rhizotube study. Both experiments constituted a first approach to characterising the root system with the aim of establishing simple methods to screen larger numbers of genotypes. Therefore, only a subset of RCSLs, contrasting in their response to drought in the field studies, was used. Nevertheless, some interesting results were obtained that could guide future studies.

4.4.1 RCSLs genotypic variation for root growth and root morphological traits

Genotypic differences were found for root growth parameters and root morphology in the RCSLs suggesting that the introgressed regions of the wild barley genome influence the root system development during the early stages of growth. Traits such as root elongation rate, change in root angle, root diameter and root length density were found to vary in genotypes with contrasting response to drought in field trials. Genotypic differences in seedling root traits may not only influence early plant establishment, but also affect nutrient and water soil capture throughout plant development (Grando and Ceccarelli, 1995; Watt *et al.*, 2006). In fact, seminal roots (emerging from the seed) of the majority of the small seeded cereals seem to play an important role in capturing deep soil resources as compared to nodal roots (emerging from the stem). In barley, seminal axes have been considered to contribute to adaptation to moisture stress environments significantly (Grando and Ceccarelli, 1995; Tyagi *et al.*, 2011). Therefore, genotypic differences in seminal root development may contribute variation in the root system architecture from early stages of development that would impact on the performance of adult plants (Tuberosa *et al.*, 2002; Manschadi *et al.*, 2007). The genotypic variation found for root traits in the RCSLs are discussed in the following sections in the context of drought stress tolerance.

4.4.1.1 Genotypic variation for seminal roots elongation rate and root angle

Genotypic variation observed in the pouch experiment for seminal root growth rate and root angle could suggest differential rooting depth and root length density across the RCSLs. Segregation for these two traits has been found to define the deep rooting phenotype in cereals (Araki *et al.*, 2002). In this study the authors observed that greater root elongation rate and increased gravitropism were associated with increased photo-assimilates translocated to root

tips, which would promote vertical root growth for longer in upland rice. Therefore genotypic variations for seminal root elongation rate and root angle could be advantageous for improved drought tolerance in water-restricted environments since these traits could potentially facilitate access to deep soil moisture.

Previous work with the RCSLs would support this hypothesis. Del Pozo *et al.*, (2012) suggested segregation for the deep root phenotype within the RCSLs population. The authors attributed increased values of grain $\Delta^{13}C$ in drought tolerant RCSLs to a greater access to soil water during grain filling and a more extensive root system which aid the maintenance of high yields in the Mediterranean conditions of the experiment (Tambussi *et al.*, 2007).

However, the number of lines tested in the present study was limited and those with potentially enhanced deep rooting phenotype as a consequence of increased root length and root angle at the seedling stage were those that experienced larger yield penalty as a consequence of water deficit in the field. This is the case of OSU144, which showed a vigorous root system with increased root elongation rate and root angle variation in the pouches and large root length density when grown in field soil in rhizotubes. Despite reaching high yield potential values, this genotype was consistently sensitive to water stress in field trials. In 2013 for instance, it suffered a 36.9% reduction in yield, one of the largest reductions amongst the RCSLs.

4.4.1.2 Putative role of mechanisms regulating water use aboveground

Even though deep rooting may facilitate the access to soil moisture, other physiological and developmental traits would determine the effectiveness of the water use aboveground, which will also depend on the environmental conditions. For example, the genotypic variation for RCSLs stomatal conductance suggested by Mendez *et al.* (2011) and del Pozo *et al.* (2012) would determine differences in the transpiration efficiency across the population. It may be that RCSLs with an improved access to soil water and increased stomatal conductance had increased water use which, on one hand would have promoted greater yield potential but on the other would have diminished water use efficiency (WUE) and hence growth and production under drought.

Interestingly, differences in this regard were found in the rice mapping population originated from cv. Bala and cv. Azucena (Price *et al.*, 2000), two cultivars differing in shoot and root traits with potential to confer adaptability to droughted environments. The former possess shallow root system but enhanced shoot mechanisms to increase WUE under drought such as better control over stomata conductance and osmotic adjustment, whereas the latter shows

vigorous root system able to grow deep in the soil profile, which leads to increased access to soil moisture under drought (Price *et al.*, 2002). In this study, shoot traits appear to be more important than a vigorous root and deep root system for avoiding drought. In fact, it was suggested that an increased access to soil moisture but less conservative use of water aboveground increased drought susceptibility due to a quicker depletion of soil water resources in lines with increased shoot biomass.

Speculating, this could explain the results observed for OSU144 and OSU052, both sensitive to drought but high-yield potential lines. Despite having an extended root system which allows sustaining high yield values and biomass in favourable conditions, the relaxed regulation of stomatal conductance and an excess of evapotranspiration under drought would have been detrimental in water limited conditions. However, to test this hypothesis, further investigations would be required.

Hence, depending on the environment, shoot traits sustaining a conservative use of water aboveground can be more important to maintaining yield in drying soils rather than root traits conferring adaptability and better access to soil water in water stress conditions such as deep root systems. Therefore, both aspects should be considered to understand water use efficiency, or the effective use of water as proposed by Blum (2011).

4.4.1.3 Large plasticity in the lateral root system formation

Large variations were found at the genotypic level for branching rate and lateral root elongation rate in the pouch experiment. Despite the homogeneity of the growing conditions in pouches, the coefficient of variation for these traits was large as a consequence of the stochastic development of root system which is especially accentuated in the behaviour of lateral roots (Forde, 2009). Statistical characterisation of root traits and growth parameters usually requires high replication numbers (Adu *et al.*, 2014), therefore it could be that the replicates used in this study were insufficient to evaluate traits with an intrinsically plastic development such as those defining lateral roots formation.

Nevertheless, OSU048 appears to have superior growth of lateral roots compared to the other RCSLs studied in the pouch experiment. An extended lateral root system would allow increased soil exploration and uptake of water and immobile nutrient such as phosphorus (Lambers *et al.*, 2006). In rice, Niones *et al.* (2015) found that the significant increased root surface area as a consequence of increased development of lateral roots, conferred adaptability to drought and soil moisture fluctuating environments. Similarly, in maize Zhan *et al.* (2015) found that

increased elongation of lateral roots was beneficial to improve water acquisition under stress. The authors found that fewer lateral roots but longer (increased lateral elongation rate) would reduce the metabolic costs of lateral root formation and increase water uptake efficiently compared to the root phenotype with several lateral roots but short (increased branching rate). In both studies, the development of lateral roots was correlated with increased shoot dry matter production under drought.

Thus, since OSU048 was found to actively develop longer lateral roots in the pouch experiment in relation to the other genotypes, it is tempting to speculate about the increased root surface area found for this genotype under drought in rhizotubes. It could be that increased elongation of lateral roots under stress augmented the root surface area and so, water uptake in OSU048. This adaptive response would have contributed to maintaining stable shoot growth under drought contrasting with the response observed in the other two genotypes where root surface and shoot dry matter were reduced under drought. These assumptions would require validation in further investigations.

4.4.1.4 Root diameter variations detected in both experiments

Genotypic variation for root diameter was identified in the pouches and rhizotubes. Root diameters were larger in pouches than in soil and, contrary to the observations in the pouch experiment, OSU048 showed thinner roots on average compared to OSU144 when grown in soil.

The confounding effects of an extended lateral root system in the rhizotubes or the differential behaviour of roots when grown in different growing media could explain the dissimilarities between experiments. Similar results were reported by Hargreaves *et al.* (2009) in a study on barley seedlings grown in 2D gel chambers and pots filled with soil. Root diameters were larger in gel chambers and the genotypic differences found for root diameter varied markedly in different growing media whereas other traits such as root angle gave similar results across both experiments.

Considering all of these points, in the present study the exotic genome seems to contribute increases in the RCSLs root diameter since cv. Harrington exhibited consistently thinner roots compared to the RCSLs studied in the two experiments. It is interesting to note that the development of finer roots in modern cultivars seems to be coupled with an increased specific root length as observed by Bertholdsson and Brantestam (2009) using a collection of Scandinavian barley landraces, old and new varieties. This trait would favour resource capture

at shallow depths (King *et al.*, 2003) which indeed has been found advantageous for increasing soil volume exploration and phosphorus uptake in modern barley cultivars grown in non-restrictive environments (Gahoonia and Nielsen, 2004). Still, little is known about the benefits of this phenotype under more restrictive environmental conditions where plants depend on deep stored moisture.

Larger root diameters seem to favour root vertical growth compared to thin roots, promoting increased deep root ratio in the soil profile as it has been observed in rice (Araki *et al.*, 2002; Kato *et al.*, 2006; Clark *et al.*, 2008). Despite the fact that the narrow rhizotubes used in this study did not allow testing this hypothesis, variation in root diameter could also be an indication of the differential deep rooting ability in the RCSLs.

Water deficit in the soil profile increases soil mechanical impedance, being a major limitation for root growth and expansion (Cairns *et al.*, 2004; Bengough *et al.*, 2011). Under this physical restriction, thicker roots penetrate better in drying impeded soils being able to access deep soil moisture. An increased root diameter seems to release some pressure forces in the root elongation zone allowing thicker roots to grow better under restrictive conditions (Materechera *et al.*, 1991; Kirby and Bengough, 2002; Bengough *et al.*, 2006). In addition, positive correlations have been found between root thickness and root angle since larger diameters seem to favour root penetration deeper in the soil profile (Araki *et al.*, 2002; Clark *et al.*, 2008).

However, the deep rooting phenotype is a complex trait that would depend on several anatomical root features in addition to just root diameter. For example, the mucilagenous substances of the rhizosheath would aid to reduce the friction on the root–soil interface, which would favour root growth in dry soils (Kirby and Bengough, 2002). Also, the development of root hairs has been associated with improved root anchoring and penetration in mechanically impeded soils as observed by Haling *et al.* (2013) in barley. Further investigations with the RCSLs population would be needed to understand the genotypic variation for root diameter and its implication in the root system architecture.

4.4.1.5 Increased root length density in cv. Harrington

Genotypic differences in root length density were found in the rhizotubes experiment. Harrington and OSU144 were found superior compared to OSU048 for this trait. In the case of the elite cultivar, the increased root length per unit of soil concurred with a reduced average root diameter and possibly with an extended root system size since both traits seem to be directly related (Palta *et al.*, 2011). Increased root system size has been indirectly favoured in the

development of elite varieties adapted to high input environments (Chloupek *et al.*, 2006; Bertholdsson and Brantestam, 2009) which appears to be advantageous for coping with moderate drought stress conditions (Chloupek *et al.*, 2010). Therefore, the moderate yield reduction as a consequence of water shortage in cv. Harrington (up to 24.7% in 2013) could be associated with an optimum root system size for avoiding mild drought stress.

4.4.1.6 Variation in seminal root vigour

The genotypic differences in the RCSLs root traits (root elongation rate, root diameter and root length density) could represent differential seedling vigour, supporting the evidence observed by Inostroza *et al.* (2011). In this work the authors evaluated the growth of 80 RCSLs seedlings focusing on shoot developmental traits. RCSLs with increased seedling vigour were identified, although no relationship with yield under drought was found. Seedling vigour has been considered as an important selection criteria for water use efficiency (WUE) and drought tolerance in cereal crops (Condon *et al.*, 2004; Bertholdsson and Brantestam, 2009), particularly for ensuring seedling establishment.

In the present study OSU048 seedlings were found to have reduced shoot dry matter accumulation compared to their root biomass in the pouch experiment. The water use of this low vigour line was presumably reduced compared to the other genotypes, which would have increased its water use efficiency. This could explain the stable performance across water treatments in the field despite the fact that moderate water use did not result in high yields. However, in this case we could also hypothesize that the segregation for loci controlling plant phenology in the RCSLs could have affected seedlings growth vigour (see section 2.4.1.2). The contrasting growth habit of the parents of the population, spring and ancestral winter habit (Karsai *et al.*, 2004), led to large differences in flowering time among the RCSLs (see section 3.4.2). Despite the fact that loci controlling the vernalisation response were not found associated with heading date, genotypes such as OSU048 flowered significantly later than cv. Harrington (see section 2.3.2.3). This line possesses exotic chromosome introgressions at two loci controlling vernalization response in barley, *Vrn-H2* and *Vrn-H3* on chromosomes 4H and 7H respectively which have been found to exert effects on seedling growth habit (Boyd *et al.*, 2003). Therefore it could be that OSU048 had a moderate vernalisation requirement and that its diminished early vigour is associated with the prostrate growth habit characteristic of the ancestral winter barley phenotype of the donor parent (see section 3.4.2).

4.4.2 Pouches and rhizotubes were found suitable for phenotyping the RCSLs root system

The selection of an appropriate root phenotyping approach would depend on the root parameters targeted, the level of throughput required and the sample size (Downie *et al.*, 2015). For example, 2D pouch experiments, as used in the present study, constitute a non-destructive and low-cost approach that allows the evaluation of root dynamic growth *in vivo* in a large number of individuals. Even though the studies are restricted to seedlings root growth in a thin layer, the time-lapse images obtained with scanners or cameras allow real-time monitoring as the root develops (Hund *et al.*, 2009b; Adu *et al.*, 2014). In addition this platform offers an homogeneous growing media especially suitable for genetic studies aiming to dissect the genetic components controlling root performance (Atkinson *et al.*, 2015; Thomas *et al.*, 2016).

In the present study, despite the limited number of genotypes, genotypic differences for important root traits were detected between groups of lines with contrasted response to water deficit in the field. Further investigations using the 28 RCSLs evaluated in chapters 2 and 3 would aid the identification of chromosome regions associated with the variation found for elongation rate of seminal and lateral roots and gravitropic rate. In addition, further experiments would help to identify the association of major developmental QTLs with the performance of root system during early growth. However, improvements in automating the pipeline for image processing, similarly to one used in the studies conducted in the wheat double haploid population derived from cv. Savannah × cv. Rialto (Atkinson *et al.*, 2015) and a large group of oil seed rape genotypes (Thomas *et al.*, 2016), would be required. Also, a larger number of replicates would need to be considered, particularly in light of the large phenotypic variability of the lateral root system observed in this study.

Alternatively, the rhizotube experiment offered the opportunity to test the RCSLs root system development in field soil. Contrary to the pouch experiment, this is a destructive method that requires sampling the roots to assess their phenotype and is labour intensive in terms of sample processing. The main advantage of this approach is that it brings the opportunity of applying different treatments to test root traits adaptability in a more realistic environment for the roots. For example, Karley *et al.* (2011) used this method to assess the effect of nitrogen acquisition in a group of old barley cultivars and modern semi-dwarfing varieties. In this study, the authors used a stacked sampling to evaluate the development of the roots at different growth stages of the crop. Although, genotypic differences were identified, the growth stage of the plant was not significantly associated with root development. The practicalities of this laborious method complicate establishing large-scale experiments in a larger number of genotypes and replicates. However, root phenotyping technologies are constantly progressing and new optimised and

automated versions of this methodology seem to be suitable not only for high-throughput root phenotyping but also for monitoring root growth in three dimension systems.

X-ray computed tomography (CT) for example, allows detailed root architecture examination in 3D (Tracy *et al.*, 2010). This high-throughput technology can be applied to the study of root system dynamics and it has been successfully used for example in wheat (Tracy *et al.*, 2012) and rice (Zappala *et al.*, 2013). However, this method is costly and to date has only been used on small numbers of individuals and the image noise derived from differences in the moisture content in the soil column complicates the interpretation of the results (Zappala *et al.*, 2013). A newly released root phenotyping platform called RhizoTubes (Jeudy *et al.*, 2016) could possibly be more suitable for assessing large numbers of individuals in the same experiment. This system allows evaluating 1200 rhizotubes at a time in a high-throughput system that simplifies the extraction of root and shoots parameters in an automated process. This platform has been tested in different crop species in limiting and not-limiting conditions and may be a useful approach for breeding programmes to identify genetic variation for key root traits that could confer adaptability to environmental constrains. However, phenotyping effectively the hidden half of plants is not only about technology but also the scientist mind's eye to develop low-cost approaches that enable relatively large throughout in the identification of new sources of genetic variation, if possible, in field conditions. In this regard a simple and striking approach is the 'basket' method developed by Oyanagi *et al.* (1993) by which the root growth angle of cereals as wheat and rice can be effectively characterised, either in the field or controlled experimental conditions, by the position where roots arise from the basket mesh where a single plant is grown (Kato *et al.*, 2006; Uga, Okuno and Yano, 2011). Other interesting approaches involve a layer of herbicide buried in rhizoboxes where plants are grown and scored depending on the herbicide symptoms showed aboveground, which allows assessing the deep rooting capacity of seedlings (Al-Shugeairy *et al.*, 2014).

In summary, this preliminary study describes two approaches to phenotype the RCSLs root system development showing the potential of the wild barley genome to contribute genetic variation for enhanced root system performance during the early stages of crop development. The genotypic variation found for root elongation rate, root diameter and root depth density seem to respond to differences in the RCSLs root system vigour, which has been found a beneficial trait for improved seedling establishment and enhanced access to soil moisture under stress conditions in cereal crops. These results are in agreement with previous observations in the RCSLs population. However, it is still unclear how traits such as increased rooting depth could enhance water use efficiency while having a positive impact on yield production under water-limited conditions in the RCSLs. Further studies using the pouch experimental approach

in the 28 RCSLs used in previous chapters would be recommended for detecting chromosome regions underlying the genetic variation found for important root architectural traits and the effect of major developmental genes on the root system growth.

4.5 Conclusion

The introgressed chromosome region of the wild barley accession Caesarea 26-24 induced morphological and developmental changes in the RCSLs root system from early growth stages of development compared to cv. Harrington. The selection of lines with contrasting response to water deficit in the field revealed genotypic variations for root growth parameters and morphology in seedlings examined in controlled conditions. The 2D pouch experimental approach was found appropriate for obtaining time lapse data and to characterise the root growth in the RCSLs seedlings. Also, the rhizotubes trial was found suitable for confirming some genotypic differences found in the pouches when the seedlings were grown in field soil; however a larger number of replicates would be needed in this case to determine possible adjustments of root system development in response to the water available.

Segregation for seminal roots elongation rate and root diameter from early stages of development could be associated with variations in the deep rooting phenotype in the RCSLs which could be beneficial for coping with water stress in specific environments. This observation would agree with evidence found in previous studies where the carbon isotope discrimination in the grain of droughted plants evaluated in a Mediterranean environment was associated with a deeper rooting system during the grain filling period (del Pozo *et al.*, 2012). However, it was not possible to correlate the deeper root phenotype with better performance under drought for the experimental conditions of the present study. Genotypes such as OSU144 were sensitive in their response to water deficit in the field despite the evidence here suggesting the potential increase of root growth in depth from seedling development. Genotypic variation for traits determining the efficiency in the water use aboveground, such as the stomata conductance and the osmotic adjustment, should be taken into account in future studies in order to understand the effect of improved water accessibility by the roots on the performance of the crop in target environments. Nevertheless, greater root vigour (increased elongation and root length density) seems to respond to differences in the RCSLs seminal vigour (Inostroza *et al.*, 2011) which seems to be related to increased yield potential in the RCSLs assessed in this chapter.

The study set up the basis for root system evaluations on a larger number of RCSLs using a simple and optimised root phenotyping approach. This would aid not only the identification of chromosome regions controlling the variation observed for specific root traits with potential to increase adaptability to water deficit conditions, but also the understanding of the indirect effect of modern breeding on the selection of root traits favouring adaptation to high-input environments.

5 General discussion and conclusions

Given the amount of exotic genetic resources with potential value for barley crop improvement and the imperative need for enriching the crop genetic base, effective pre-breeding strategies to systematically mine novel allelic variation are required (Weichert *et al.*, 2010). Today, with the implementation of high-throughput genotyping platforms and the advances towards whole genome sequencing data, the identification of novel functional alleles and eventually the causative genes responsible for quantitative variation of economically important traits is becoming more easily attainable. However, the process of "rewilding" or utilisation of exotic genomes in breeding programmes is still hampered by two major bottlenecks: the generalised reduced fitness of the progeny of wide crosses as a consequence of "linkage drag" and the acquisition of precise high-quality phenotypic data on which QTL mapping success is dependant (Feuillet *et al.*, 2008; Myles *et al.*, 2009).

In this context, one possible approach to overcome these trade-offs is the utilisation of advanced backcross populations such as the Recombinant Chromosome Substitution Lines (RCSLs) (Matus *et al.*, 2003). Although this approach limits the scope of genotypic and phenotypic variation to the two parents of the population, the occurrence of deleterious alleles associated with the unadapted genome is significantly reduced, facilitating the detection of low frequency functional alleles. Also groups of Near Isogenic Lines (NILs) can be derived directly from these for QTL fine-mapping and cloning. In addition, the establishment of exotic introgression libraries derived from the original cross allows optimising QTL mapping studies of more detailed phenotypic evaluations either from multi-environment trial experiments or high-throughput phenotyping platforms (Zamir, 2001).

Insights from previous studies showed the potential of the RCSLs population developed by Matus et al. (2003) for mining new exotic allelic variation for barley crop improved adaptation to drought environments (Inostroza *et al.*, 2007, 2009; Mendez *et al.*, 2011; del Pozo *et al.*, 2012). In light of these observations, the present work attempts to identify advantageous alleles from the wild barley genome that could potentially contribute to crop improved performance and adaptation. The study focused on a set of 29 RCSLs representing the genome of the wild barley accession, Caesarea 26-24, in the genetic background of the elite North American spring malting barley, cv. Harrington. Field trials conducted in rain-out shelters over two growing seasons provided a precise phenotypic characterisation of the RCSLs performance and its response to water limited conditions (Chapter 2). The data gathered established the basis for conducting an association analysis designed to identify the chromosomal regions involved in the RCSLs phenotypic variation for relevant morphological, developmental and agronomic traits. In addition, the high-density genotypic characterisation achieved with the 9K iSelect SNP platform

(Comadran *et al.*, 2012) provided a detailed estimation of the extent of the wild genome introgressions, which could be further analysed using the new barley genome assembly (IBSC 2016) to identify causative genes of some target QTLs (Chapter 3). Finally, phenotypic evaluations of the RCSLs root system in controlled experimental conditions attempted to identify genotypic variation for key root architectural traits that could be beneficial for increased water uptake under water deficit regimes (Chapter 4).

5.1 Wild barley germplasm for improving barley crop yield performance and stability

Evidence was found to suggest that the wild barley genome contributes favourable alleles to increase the grain sink strength of the elite cultivar cv. Harrington. Since further genetic gains on yield and yield stability will largely rely on yield-component traits directly controlling crop source and sink relationships (Coventry *et al.*, 2003; Araus *et al.*, 2008; White *et al.*, 2015), this result was considered an interesting outcome of the present study. In Chapter 2, the large phenotypic variation found for thousand grain weight (TGW) and the overall superiority of the RCSLs compared to cv. Harrington was in agreement with previous observations by Matus *et al.* (2003). The genetic dissection of the trait in the association analysis conducted in Chapter 3 revealed putative QTLs on chromosomes 3H and 5H associated with grain weight and size that would constitutively increase its performance. These associations were particularly interesting since they were not linked to yield penalties or developmental loci.

The effective translocation and accumulation of stored reserves to the forming grain in wild barleys could be seen as an adaptive mechanism to ensure its reproductive success in dry natural habitats (Chaves and Oliveira, 2004; Blum, 2005; Passioura, 2007). In the RCSLs, the introgression of genes associated with these mechanisms could have directly optimised the ability to remobilise and accumulate photo-assimilates from the source organs to the grain (i.e. reproductive sinks), favouring the development of bigger and heavier grains in the cultivated barley.

Three interesting candidate genes involved in the transmembrane transport of sugar and in the regulation pathway of starch biosynthesis of developing grains were identified as putative causative genes underlying these QTLs. The latter encodes a 14-3-3 protein which has been directly involved in the regulation of the starch biosynthesis in Arabidopsis (Sehnke *et al.*, 2001) and in cereal crops (Zhang *et al.*, 2014; Yu *et al.*, 2016). Indeed Alexander and Morris (2006) showed how increased levels of 14-3-3 protein in barley endosperms inhibited the activity of sucrose synthase proteins which are responsible for the storage of this polysaccharide in sink tissues. Further investigations would be required to determine whether the exotic allelic variant of these genes actually contributed differences in sucrose transport and starch accumulation in the grains of the RCSLs with these introgressions. If that was the case, this

would be a promising candidate to adjust source and sink relationships in cultivated barley by favouring increases in the ability of accumulating photo-assimilates in the grain (i.e. sink strength) which would potentially contribute direct genetic gains not only on yield but also in yield stability (Alexander and Morris, 2006; Weichert *et al.*, 2010; White *et al.*, 2015).

Indeed, the stability statistic analyses conducted in Chapter 2 highlighted the positive contribution of the wild barley genome in optimising crop stability, particularly in regard to its quality (estimated as grain weight or TGW). Although investigating the genetics underlying the stability in crop performance was beyond the scope of the present study, we could hypothesise that the efficient allocation of carbohydrates in the grain could have contributed to maintaining its weight and size stable across different environmental conditions. This would not only lead to more reliable crop production but would also help to reduce the yield gap between optimum and stress condition (Cattivelli *et al.*, 2008).

Other morpho-physiological aspects, such as the reduced tillering capacity of the RCSLs and the presence of epicuticular waxes in the spike, could be seen as adaptive traits responsible for sustained grain weight and crop performance in water-limited environments (García Del Moral *et al.*, 2003; Abebe *et al.*, 2010). Even though under the conditions of the present study it was unclear as to what the role of epicuticular waxes were in the maintenance of grain weight and yield, other authors have found that genotypes lacking in epicuticular waxes may be more susceptible to drought than glaucous lines. Febrero (1998) associated the larger grain $\Delta^{13}C$ values of glaucous (non-glossy) barley with reduced transpiration efficiency during grain filling and a superior performance under drought due to an increased stomatal conductance and photosynthesis rate of these lines. In this case spike, peduncle and leaf sheath were glaucous, which could possibly enhance the differences as compared to the non-glaucous line. It would be interesting to investigate whether the differences found for grain $\Delta^{13}C$ in RCSLs with superior performance in Mediterranean environments relate to the spike wax composition and not only with access to deep soil moisture as the authors suggested (del Pozo *et al.*, 2012).

In conclusion, novel genotypic variation from a wild barley accession may contribute adjustments in crop source and sink relationships, which could directly lead to genetic gains in yield and yield stability. Chromosome regions and candidate genes with potential to constitutively enhance the translocation and accumulation of photo-assimilates in the grain have been identified. In these regions the wild barley alleles could contribute to optimise sink strength of the crop by boosting the potential size of individual grains. Further research on the efficient translocation and accumulation of starch in the grains endosperm would be required to confirm this assumption. In parallel, sequencing the candidate genes and distinguish haplotype variants in the population would be recommended. Likewise, additional research to specify the

precise role of epicuticular waxes in the protection of spike photosynthetic structures and its contribution to maintain grain weight stable may be of interest for improved crop resiliency.

5.2 Exotic genetic libraries in the detection of novel genetic variants

This study supports the utilisation of exotic genetic libraries composed of a minimum set of genetically unique lines for conducting precise phenotyping studies that would not be attainable otherwise. In this case, the group of RCSLs selected to represent the entire genome of the wild donor parent, Caesarea 26-24, were used in field trials where precise control over the water treatments was required.

Accurate identification of QTLs from a small group of RCSL genotypes is challenging, especially in early backcross lines which contain several alien inserts in their genome. However, being able to target lines and chromosome regions in earlier generations for the development of NILs would contribute to simplify the process of detection of novel genetic variability for the crop. Other studies based on barley exotic genetic libraries estimate QTL locations in target segment making the introgression lines from a BC3 or BC4 generation significantly different from the donor parent (Hori *et al.*, 2005; Schmalenbach *et al.*, 2009). This approach is quite effective in groups of lines with a single introgression; however, the establishment of such populations and the rounds of phenotypic and genotypic characterisation can be time consuming and laborious.

In this context, the mixed model framework provided the power and flexibility necessary to conduct a QTL mapping study on field trial data. This is the first study in an exotic genetic library using this approach; however, different authors have highlighted the benefits of this method for QTL-mapping, especially for multi-environment trial data (Malosetti *et al.*, 2008; Bauer *et al.*, 2009; van Eeuwijk *et al.*, 2010). In Chapter 3, REML single-locus analysis was used to detect marker–trait associations. Even though the method gives a coarse estimation of the QTLs, it was found suitable to detect exotic alleles that improved the performance of relevant quantitative traits. Nevertheless, it should be noted that the abundance of marker–trait associations detected with the REML single-locus approach needs to be taken cautiously since it could entail some overestimations that may confound the results of the analysis (Bauer *et al.*, 2009), some possibly as a result of epistatic interaction between wild barley introgressions (von Korff *et al.*, 2010). Only by breaking up the exotic introgressions through new backcrosses can a more accurate location of the QTLs be achieved.

Key to the association study conducted was the use of high resolution genotypic characterisation of the RCSLs with the Infinium 9K iSelect SNP platform (Comadran *et al.*, 2012). This not only allowed an accurate estimation of the extent of the wild introgressed regions compared to previous characterisations (GCP, 2010) (BOPA1), but also the identification of candidate genes

in the new barley genome assembly (IBSC, 2016). In addition, the enhanced marker genome coverage meant that the 29 RCSLs were amenable for QTL fine-mapping of qualitatively measurable characters such as the grain threshability locus *thresh-1* and the novel QTL associated with the glossy phenotype of the spike GLS1. The former was initially fine mapped by Schmalenbach *et al.* (2011) using large set of introgression lines (73 $BC_3S_{4:6}$ and 91 BC_4S_2) genotyped with the 1536-SNP barley BOPA1 set. These results show the potential of this group of lines for optimising QTL mapping of simple and complex traits controlled by few loci and relatively great heritability.

In addition, given the need to investigate new secondary traits that could directly contribute crop stability and sustainability, the RCSLs constitute an important genetic resource to infer novel strategies for crop adaptation. In fact, the genotypic differences in the RCSLs rhizosheath (T. George, personal communication) and root-C deposition (Mwafulirwa *et al.*, 2016) have found desirable traits to contribute to agricultural sustainability. Also, genotypic variation for other traits such as grain $\Delta^{13}C$, which is a good indicator of plant photosynthetic performance, has been associated with the RCSLs and their improved performance under drought (del Pozo *et al.*, 2012). Generally these traits are costly to measure since they require isotope labelling and sophisticated estimations. Therefore, a manageable group of lines is essential to dissect the quantitative inheritance of these characters.

To sum up, the present study provides the basis for conducting QTL mapping studies using a small set of RCSLs. The high-throughput genotypic characterisation of the lines and the design of an effective marker–trait association analysis have led to the identification of novel exotic QTLs that could be deployed in a MAS programme for crop improvement; however it has been highlighted that the need for a less conservative statistical approach requires caution in the interpretation of the results. In this regard, screening for candidate genes in the new barley genome assembly and seeking homologies in other plant genomes was indispensable to support the findings of the present study. The utilisation of the NILs available at the JHI (BC_3S_2) for these lines would provide a better estimate of the QTL effects as well as the means to characterise the genes underlying the phenotypic variations at these loci. Also investigating this variation in different elite genetic backgrounds would be recommended. Finally, the development of a new introgression library based on advanced generation could enhance the accuracy of QTL detection.

5.3 Root system traits for improved water acquisition

The large genotypic variation observed in quantitative traits aboveground (Chapter 2) was also encountered belowground. In Chapter 4, RCSLs with contrasted response to water deficit in the field were found to diverge for root system traits that could potentially optimise root soil exploration in scarce water environments. These observations were made in a controlled 2D

pouch experimental platform designed specifically for the present study. Genotypic variation was found for seedlings seminal root elongation rate, root gravitropism and root diameter, suggesting differential rooting depth ability in the RCSLs (Araki *et al.*, 2002; Steele *et al.*, 2013). This quantitative trait is known to alleviate the effects of water deficit in performance of cereal crops (Kato *et al.*, 2006; Hund *et al.,* 2009a; Lopes and Reynolds, 2010). In fact, deeper root phenotypes have been found to favour yield under drought in rice by optimising grain filling and enhancing grain weight of droughted plants, hence contributing to yield stability (Arai-Sanoh *et al.*, 2014).

However from the results of the present study, it was unclear whether this phenotype could relate to improved performance under water deficit. Interestingly increased root vigour was found associated with genotypes with greater yield potential. Nonetheless, previous evaluations in Mediterranean environments suggested that genotypic differences in the RCSLs deep rooting capacity were in accordance with the large values of grain $\Delta^{13}C$ in RCSLs with improved drought tolerance (del Pozo *et al.*, 2012). In light of the results of the present study, it would be interesting to elucidate whether these differences also responded to variations in the photosynthetic capacity of the spike and whether increases in grain weight maintained the yield with increased drought tolerance in Mediterranean environments.

In parallel to the results obtained for the present study, this first evaluation of the RCSLs root system was used in an integrated pipeline aimed at optimising the detection of root growth QTLs within the framework of a model-based mathematical approach (de la Fuente Cantó *et al.,* 2016 in prep, Appendix 20). This work was the result of collaboration with modellers participating in the EURooT project based at The James Hutton Institute and constitutes a novel approach designed to facilitate the understanding of root system dynamic development and its genetic basis. Using six genotypes as a case study, the methodological framework was established in three main steps: 1) root phenotyping in a 2D pouch experiment suitable for obtaining time-lapse root imaging data; 2) optimisation algorithm for model-based extraction of root growth parameters; and 3) implementation of the novel combinatorial QTL mapping approach (C-QTL) for quick identification of chromosome regions that contribute the most to variations in root system development.

The new C-QTL mapping theoretical framework was developed in view of the genotypic architecture of the RCSLs. Due to the fact that the RCSLs harbour several wild barley chromosome introgressions (six on average) with a range of different overlaps, a simple test carried out in other groups of introgression lines with single alien segments cannot be adopted. Neither could the mixed model approach be applied. Therefore, a method based on grouping lines according to their phenotypic similarity was developed. Briefly, markers were scored in a computed metric according to variation between and within groups of similar lines. Since there

are many different ways of grouping genotypes, a cluster algorithm was used to create the sets of relevant groups on which the metric is cumulated. As a result, the markers scoring outlines regions of the genome that may be linked to variation in a quantitative trait, regions of the genome that vary without having an effect on a quantitative trait, and regions of the genome on which no information can be derived.

Heading date for the 29 RCSLs was used as a reference trait to validate this approach. Major chromosome regions associated with flowering time and segregating in the population were detected. However, since root growth was uniquely characterised for 5 RCSLs, coarse associations were found for root quantitative traits. These were more substantial for elongation rate of seminal and lateral roots whereas weaker associations were found for gravitropic and branching rate. Although some aspects of the method are still unclear and additional theoretical work is required, this approach appears promising since it could facilitate a "first glance" location of marker–trait associations for complex traits in groups of early generations of introgression lines with an unbalanced genotypic constitution. This could contribute to optimise the time and resources invested in MAS backcross breeding programmes and the selection of lines for advanced progenies and development of NILs for QTL fine-mapping, particularly for traits that require laborious and time-consuming analyses.

To conclude, the RCSLs genotypic variation for root architectural traits related to drought adaptation could have determined the performance of adult plants in water scarce environments. New approaches integrating root models to QTL mapping studies could contribute to identify the chromosome regions associated with this variation. Further investigations using the RCSLs exotic library would need to be conducted to effectively dissect the genetic basis of these traits. Finally, the C-QTL mapping approach has the potential for optimising the resources invested in the study of complex quantitative traits such as those related to the root system growth.

6 Future prospects

Advances in genetic and genomic approaches are facilitating the access to the natural genetic diversity occurring in the wild barley genepool. The enormous collection of germplasm and the large-scale genotyping platforms (i.e. iSelect SNP platforms, exome capture) are aiding the elucidation of the genetic base of barley's adaptive responses (Russell *et al.*, 2016). Also, modelling studies are useful tools to predict new climatic scenarios for which specific crop adaptive strategies need to be favoured (Dawson *et al.*, 2015). In addition, the development of new genetic resources such as the Nested Association Mapping (NAM) population of wild barley, HEB-25 (1460 BC_1S_3 lines belonging to 25 families derived from 25 wild donor accessions and the same cultivar recurrent parent) appears highly promising to seek new exotic allelic variants with utility for the cultivated genepool in genome wide scan studies (Maurer *et al.*, 2015). These tools allow large genetic screenings for favourable alleles that can be incorporated into the cultivated genepool (Saade *et al.*, 2016). However, small-scale experiments are also required to investigate the usefulness of the new exotic genetic variation in crop performance and investigate novel secondary traits with direct impact on crop production and sustainability (Teulat *et al.*, 2002; Honsdorf *et al.*, 2014a; Mwafulirwa *et al.*, 2016). In this context, groups of lines such as the RCSLs can benefit from the high-throughput genotyping and precise phenotyping studies to underpin the improvement of the crop.

The study conducted here is an attempt to optimise the mining of novel allelic variation using a set of genotypically well-characterised RCSLs representing the genome of the drought tolerant wild accession Caesarea 26-24. From the results obtained, different aspects should be considered in current and future research:

1. **The RCSLs provide a coarse QTL location that needs to be verified by means of QTL-NILs studies**. Ongoing research is in progress to sequence and characterise target causative genes underlying the glossy spike locus (GLS1) on chromosome 1H. A set of NILs derived from one of the RCSLs (OSU060) has already been phenotyped and genotyped (C. Campoli, personal communication). This work is being conducted within the framework of the ClimBar project (ERA-NET Plus Joint Research Programming Initiative on Agriculture, Food Security and Climate Change (FACCE-JPI)) with the main goal of tagging genes in locally adapted and wild barleys that could contribute crop resilience in predicted climate change scenarios.

   Similarly, future work should concentrate on the chromosome regions putatively associated with an optimised partitioning of the photo-assimilates in the grain. The QTL on chromosome 3H is particularly interesting due to the strong candidate gene identified as a regulator of the starch biosynthesis process in the endosperm. Evaluations of the starch

accumulation in the grain of NILs derived for this region and manipulation of the gene expression, to test whether it is responsible for the favourable increased in seed plumpness and weight, would be recommended. Additionally, investigating whether this QTL confers agronomic advantage in different elite genetic backgrounds would need to be considered.

2. **The RCSLs are amenable for investigating crop adaptability to a range of environmental constraints.** The RCSLs constitute a permanent and manageable genetic resource whose, evaluation in target environments over time, would provide the means for investigating genotype by environment interactions and its genetic basis. Similar experiments to the one in the present thesis could focus on the crop responses to salinity stress or temperature. The flexibility of mixed model approaches could provide a means to model the genotypic architecture of complex traits in their response to environmental factors over different seasons. Further, with a detailed control of the climatic data it would be possible to determine the main environmental cues driving the genotype by environment interaction.

   The establishment of a new exotic genetic library based on advanced generations would be highly recommended. This would not only simplify the statistical analysis and add accuracy to the QTL detection but also it would aid to distinguish pleiotropic effects from close linkage with non-developmental QTLs and reduce the possible epistatic effects.

3. **Further investigations into the RCSLs root system architecture should be considered for mapping root QTLs that may contribute improved water and nutrient uptake efficiency.** The present study has shown the potential of the RCSL to investigate genotypic variation in root system developmental traits. In parallel, in an attempt to integrate genetic resources, high-throughput phenotyping and predictive density root models, an accelerated root phenotyping pipeline has been set up to facilitate the identification of root QTLs. Continuing with this research in the larger set of lines is highly recommended since it would not only further our understanding of the genetic control of root growth parameters in this set of lines but also it would serve as a novel proof of concept of a methodology amenable to automation for identifying root QTLs in mapping populations.

   In addition, a detailed examination of the 29 RCSLs root system architecture has been conducted by X-ray computed tomography (CT) at the University of Nottingham. The experiment has been arranged using three replicates under control conditions and the data will be soon available for a marker–trait association analysis. It would be interesting to see whether the 2D and 3D data can be used for root QTL mapping and serve as validation sets one of the other. Also, the two screening methods would provide useful data for modelling root architecture.

In summary, the RCSLs exotic genetic library has been established as a permanent genetic resource for future research, which is amenable for either field evaluations or high-throughput phenotyping experiments. In addition, the availability of near isogenic lines derived for most of the RCSLs in BC_3S_2 provide the platform for further fine-mapping of target QTLs and identification of putative functional genes. Hopefully, current and future work derived from this research will provide the means for positional cloning of QTLs with biological and agronomical interest for the development of more sustainable and resilient varieties.

7 Reference List

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8. Appendices

Appendix 1. Field trial setup 2013.

COL[1] / ROW	5	4	3	2	1
1	GUARD	GUARD	GUARD	GUARD	GUARD
2	OSU 105	OSU 124	OSU 48	OSU 47	OSU 15
3	OSU 144	OSU 51	OSU 60	Harrington	OSU 12
4	OSU 74	OSU 127	OSU 86	OSU 52	OSU 137
5	OSU 35	OSU 18	OSU 40	OSU 65	OSU 102
6	OSU 38	OSU 44	OSU 19	OSU 24	OSU 107
7	OSU 61	OSU 53	OSU 90	OSU 33	OSU 16
8	GUARD	GUARD	GUARD	GUARD	GUARD
9	GUARD	GUARD	GUARD	GUARD	GUARD
10	OSU 137	OSU 44	OSU 47	OSU 35	OSU 90
11	OSU 61	OSU 102	OSU 86	Harrington	OSU 24
12	OSU 33	OSU 38	OSU 15	OSU 60	OSU 52
13	OSU 51	OSU 16	OSU 105	OSU 19	OSU 18
14	OSU 48	OSU 65	OSU 127	OSU 53	OSU 144
15	OSU 74	OSU 40	OSU 12	OSU 107	OSU 124
16	GUARD	GUARD	GUARD	GUARD	GUARD
17	GUARD	GUARD	GUARD	GUARD	GUARD
18	OSU 137	OSU 107	OSU 60	OSU 65	OSU 105
19	OSU 90	OSU 52	OSU 102	OSU 19	OSU 127
20	OSU 53	OSU 144	OSU 74	OSU 15	OSU 44
21	OSU 12	OSU 86	OSU 18	OSU 35	OSU 33
22	OSU 24	OSU 51	OSU 47	OSU 61	OSU 40
23	OSU 38	OSU 16	OSU 48	OSU 124	Harrington
24	GUARD	GUARD	GUARD	GUARD	GUARD
25	GUARD	GUARD	GUARD	GUARD	GUARD
26	OSU 107	OSU 127	OSU 61	OSU 18	OSU 38
27	OSU 90	OSU 74	OSU 12	Harrington	OSU 65
28	OSU 124	OSU 60	OSU 44	OSU 137	OSU 86
29	OSU 105	OSU 35	OSU 52	OSU 40	OSU 53
30	OSU 102	OSU 24	OSU 48	OSU 15	OSU 51
31	OSU 33	OSU 16	OSU 19	OSU 144	OSU 47
32	GUARD	GUARD	GUARD	GUARD	GUARD

COL[1] / ROW	5	4	3	2	1
33	GUARD	GUARD	GUARD	GUARD	GUARD
34	OSU 105	OSU 61	OSU 47	OSU 24	Harrington
35	OSU 102	OSU 107	OSU 144	OSU 48	OSU 90
36	OSU 44	OSU 35	OSU 74	OSU 15	OSU 12
37	OSU 124	OSU 40	OSU 19	OSU 137	OSU 60
38	OSU 38	OSU 127	OSU 86	OSU 51	OSU 65
39	OSU 33	OSU 16	OSU 18	OSU 53	OSU 52
40	GUARD	GUARD	GUARD	GUARD	GUARD
41	GUARD	GUARD	GUARD	GUARD	GUARD
42	OSU 12	OSU 16	OSU 24	OSU 127	OSU 124
43	OSU 33	OSU 65	OSU 40	OSU 90	OSU 47
44	OSU 137	OSU 53	OSU 144	OSU 60	OSU 35
45	OSU 61	OSU 44	OSU 38	OSU 48	OSU 18
46	OSU 102	OSU 19	OSU 52	OSU 74	OSU 51
47	OSU 86	Harrington	OSU 15	OSU 105	OSU 107
48	GUARD	GUARD	GUARD	GUARD	GUARD
49	GUARD	GUARD	GUARD	GUARD	GUARD
50	OSU 86	OSU 60	OSU 24	OSU 40	OSU 53
51	OSU 48	OSU 38	OSU 107	OSU 12	OSU 19
52	Harrington	OSU 16	OSU 33	OSU 51	OSU 144
53	OSU 124	OSU 15	OSU 74	OSU 90	OSU 61
54	OSU 35	OSU 102	OSU 65	OSU 18	OSU 105
55	OSU 52	OSU 47	OSU 137	OSU 44	OSU 127
56	GUARD	GUARD	GUARD	GUARD	GUARD
57	GUARD	GUARD	GUARD	GUARD	GUARD
58	OSU 124	OSU 53	OSU 38	Harrington	OSU 74
59	OSU 51	OSU 105	OSU 90	OSU 137	OSU 16
60	OSU 61	OSU 52	OSU 35	OSU 102	OSU 40
61	OSU 144	OSU 12	OSU 24	OSU 18	OSU 86
62	OSU 65	OSU 47	OSU 19	OSU 15	OSU 48
63	OSU 60	OSU 107	OSU 33	OSU 127	OSU 44
64	GUARD	GUARD	GUARD	GUARD	GUARD

COL[1] / ROW	5	4	3	2	1
65	GUARD	GUARD	GUARD	GUARD	GUARD
66	OSU 74	OSU 144	OSU 47	OSU 65	OSU 16
67	OSU 48	OSU 52	OSU 86	OSU 60	OSU 12
68	OSU 61	OSU 38	OSU 40	OSU 35	OSU 53
69	OSU 19	OSU 102	OSU 24	OSU 127	OSU 44
70	OSU 90	Harrington	OSU 33	OSU 137	OSU 124
71	OSU 105	OSU 15	OSU 18	OSU 51	OSU 107
72	GUARD	GUARD	GUARD	GUARD	GUARD
73	GUARD	GUARD	GUARD	GUARD	GUARD
74	OSU 86	OSU 40	OSU 107	OSU 52	OSU 127
75	OSU 16	OSU 38	OSU 48	OSU 15	OSU 47
76	OSU 144	OSU 44	OSU 18	OSU 33	OSU 105
77	OSU 90	OSU 74	OSU 102	OSU 53	OSU 35
78	OSU 51	OSU 12	OSU 137	OSU 61	Harrington
79	OSU 19	OSU 65	OSU 124	OSU 60	OSU 24
80	GUARD	GUARD	GUARD	GUARD	GUARD
81	GUARD	GUARD	GUARD	GUARD	GUARD
82	OSU 124	OSU 44	OSU 61	OSU 102	OSU 86
83	OSU 15	OSU 33	OSU 24	OSU 12	OSU 74
84	OSU 127	OSU 48	OSU 90	OSU 51	OSU 38
85	OSU 144	OSU 35	OSU 107	OSU 47	OSU 60
86	OSU 19	Harrington	OSU 65	OSU 53	OSU 18
87	OSU 40	OSU 16	OSU 52	OSU 105	OSU 137
88	GUARD	GUARD	GUARD	GUARD	GUARD
89	GUARD	GUARD	GUARD	GUARD	GUARD
90	OSU 16	OSU 127	OSU 18	OSU 61	OSU 102
91	OSU 38	OSU 107	OSU 12	OSU 19	OSU 33
92	OSU 105	OSU 86	OSU 35	OSU 15	OSU 65
93	OSU 124	OSU 74	OSU 52	OSU 47	OSU 51
94	OSU 60	Harrington	OSU 90	OSU 44	OSU 40
95	OSU 24	OSU 53	OSU 144	OSU 137	OSU 48
96	GUARD	GUARD	GUARD	GUARD	GUARD

1. Rows 1 to 32 full irrigated plots; rows 33 to 64 partially irrigated plots; rows 65 to 96 no irrigated plots

Appendix 1 (continued). Field trial setup 2014

ROW \ COL[1]	5	4	3	2	1
1	GUARD	GUARD	GUARD	GUARD	GUARD
2	GUARD	GUARD	GUARD	GUARD	GUARD
3	GUARD	GUARD	GUARD	GUARD	GUARD
4	OSU 18	OSU 44	OSU 105	OSU 48	OSU 52
5	OSU 12	OSU 124	OSU 137	OSU 60	OSU 107
6	OSU 127	OSU 24	Harrington	OSU 53	OSU 90
7	OSU 86	OSU 35	OSU 38	OSU 33	OSU 40
8	OSU 51	OSU 65	OSU 47	OSU 74	OSU 19
9	OSU 144	OSU 16	OSU 61	OSU 102	OSU 15
10	GUARD	GUARD	GUARD	GUARD	GUARD
11	GUARD	GUARD	GUARD	GUARD	GUARD
12	OSU 18	OSU 33	OSU 38	OSU 35	OSU 19
13	OSU 24	OSU 137	OSU 48	OSU 144	OSU 65
14	OSU 61	OSU 86	OSU 127	OSU 107	OSU 44
15	OSU 60	OSU 51	OSU 15	OSU 105	Harrington
16	OSU 52	OSU 90	OSU 124	OSU 47	OSU 102
17	OSU 40	OSU 16	OSU 74	OSU 53	OSU 12
18	GUARD	GUARD	GUARD	GUARD	GUARD
19	GUARD	GUARD	GUARD	GUARD	GUARD
20	OSU 127	OSU 38	OSU 15	OSU 137	OSU 40
21	OSU 33	OSU 16	OSU 60	OSU 44	OSU 90
22	OSU 61	OSU 12	OSU 47	OSU 24	OSU 18
23	OSU 51	OSU 53	OSU 35	OSU 144	OSU 124
24	OSU 107	OSU 48	Harrington	OSU 19	OSU 102
25	OSU 65	OSU 52	OSU 86	OSU 74	OSU 105
26	GUARD	GUARD	GUARD	GUARD	GUARD
27	GUARD	GUARD	GUARD	GUARD	GUARD
28	OSU 107	OSU 74	OSU 18	OSU 16	Harrington
29	OSU 38	OSU 90	OSU 65	OSU 60	OSU 144
30	OSU 24	OSU 12	OSU 15	OSU 105	OSU 33
31	OSU 102	OSU 35	OSU 137	OSU 127	OSU 52
32	OSU 51	OSU 48	OSU 44	OSU 47	OSU 40
33	OSU 86	OSU 61	OSU 53	OSU 19	OSU 124
34	GUARD	GUARD	GUARD	GUARD	GUARD

ROW \ COL[1]	5	4	3	2	1
35	GUARD	GUARD	GUARD	GUARD	GUARD
36	OSU 44	OSU 86	OSU 48	OSU 18	OSU 53
37	OSU 15	OSU 52	OSU 124	OSU 102	OSU 19
38	OSU 144	OSU 33	Harrington	OSU 105	OSU 65
39	OSU 51	OSU 137	OSU 74	OSU 24	OSU 12
40	OSU 60	OSU 16	OSU 38	OSU 127	OSU 90
41	OSU 61	OSU 107	OSU 47	OSU 35	OSU 40
42	GUARD	GUARD	GUARD	GUARD	GUARD
43	GUARD	GUARD	GUARD	GUARD	GUARD
44	OSU 15	OSU 12	OSU 124	OSU 105	OSU 107
45	OSU 127	OSU 52	OSU 48	OSU 51	OSU 65
46	OSU 40	OSU 38	OSU 33	OSU 144	OSU 102
47	OSU 24	OSU 61	OSU 19	OSU 86	OSU 74
48	Harrington	OSU 16	OSU 35	OSU 90	OSU 44
49	OSU 137	OSU 18	OSU 60	OSU 47	OSU 53
50	GUARD	GUARD	GUARD	GUARD	GUARD
51	GUARD	GUARD	GUARD	GUARD	GUARD
52	OSU 51	OSU 124	OSU 47	OSU 65	OSU 16
53	OSU 19	OSU 137	OSU 44	OSU 38	OSU 105
54	OSU 144	OSU 48	OSU 24	OSU 35	OSU 52
55	OSU 107	OSU 61	OSU 127	OSU 53	OSU 40
56	OSU 18	OSU 90	OSU 33	OSU 15	OSU 74
57	Harrington	OSU 102	OSU 12	OSU 86	OSU 60
58	GUARD	GUARD	GUARD	GUARD	GUARD
59	GUARD	GUARD	GUARD	GUARD	GUARD
60	OSU 124	OSU 16	OSU 144	OSU 24	OSU 53
61	OSU 40	OSU 48	OSU 12	OSU 90	OSU 105
62	OSU 86	OSU 19	OSU 127	OSU 107	OSU 33
63	OSU 65	OSU 15	OSU 137	OSU 60	OSU 61
64	OSU 18	OSU 102	OSU 35	OSU 38	OSU 51
65	OSU 44	OSU 47	OSU 74	OSU 52	Harrington
66	GUARD	GUARD	GUARD	GUARD	GUARD

ROW \ COL[1]	5	4	3	2	1
67	GUARD	GUARD	GUARD	GUARD	GUARD
68	OSU 18	OSU 38	OSU 52	OSU 44	OSU 107
69	OSU 86	OSU 74	OSU 51	OSU 105	OSU 90
70	Harrington	OSU 144	OSU 35	OSU 102	OSU 124
71	OSU 127	OSU 16	OSU 33	OSU 60	OSU 48
72	OSU 47	OSU 24	OSU 19	OSU 65	OSU 12
73	OSU 40	OSU 15	OSU 53	OSU 137	OSU 61
74	GUARD	GUARD	GUARD	GUARD	GUARD
75	GUARD	GUARD	GUARD	GUARD	GUARD
76	OSU 47	OSU 137	OSU 33	OSU 144	OSU 107
77	OSU 60	OSU 16	OSU 61	OSU 51	OSU 102
78	OSU 38	OSU 12	OSU 86	OSU 48	OSU 19
79	OSU 124	OSU 18	Harrington	OSU 65	OSU 74
80	OSU 53	OSU 90	OSU 44	OSU 52	OSU 127
81	OSU 105	OSU 35	OSU 24	OSU 40	OSU 15
82	GUARD	GUARD	GUARD	GUARD	GUARD
83	GUARD	GUARD	GUARD	GUARD	GUARD
84	OSU 105	OSU 86	OSU 144	OSU 65	OSU 33
85	OSU 24	OSU 48	OSU 18	OSU 53	OSU 102
86	OSU 124	OSU 44	OSU 19	OSU 16	OSU 40
87	OSU 137	OSU 47	OSU 74	OSU 90	OSU 38
88	OSU 52	OSU 15	OSU 60	Harrington	OSU 12
89	OSU 127	OSU 35	OSU 61	OSU 107	OSU 51
90	GUARD	GUARD	GUARD	GUARD	GUARD
91	GUARD	GUARD	GUARD	GUARD	GUARD
92	OSU 127	OSU 35	OSU 38	OSU 65	OSU 61
93	OSU 53	OSU 60	OSU 137	OSU 124	OSU 86
94	OSU 44	OSU 24	Harrington	OSU 51	OSU 47
95	OSU 144	OSU 90	OSU 19	OSU 15	OSU 102
96	OSU 12	OSU 107	OSU 105	OSU 18	OSU 16
97	OSU 74	OSU 40	OSU 48	OSU 33	OSU 52
98	GUARD	GUARD	GUARD	GUARD	GUARD
99	GUARD	GUARD	GUARD	GUARD	GUARD
100	GUARD	GUARD	GUARD	GUARD	GUARD

1. Rows 1 to 34 non irrigated plots plots; rows 35 to 66 partially irrigated plots; rows 67 to 100 full irrigated plots

Appendix 2- GenStat scripts Chapter 2. (A) Three factorial mixed model and (B) two factorial mixed model (B) to assess the effect of the water treatments on the RCSLs performance considering two or one growing season respectively.

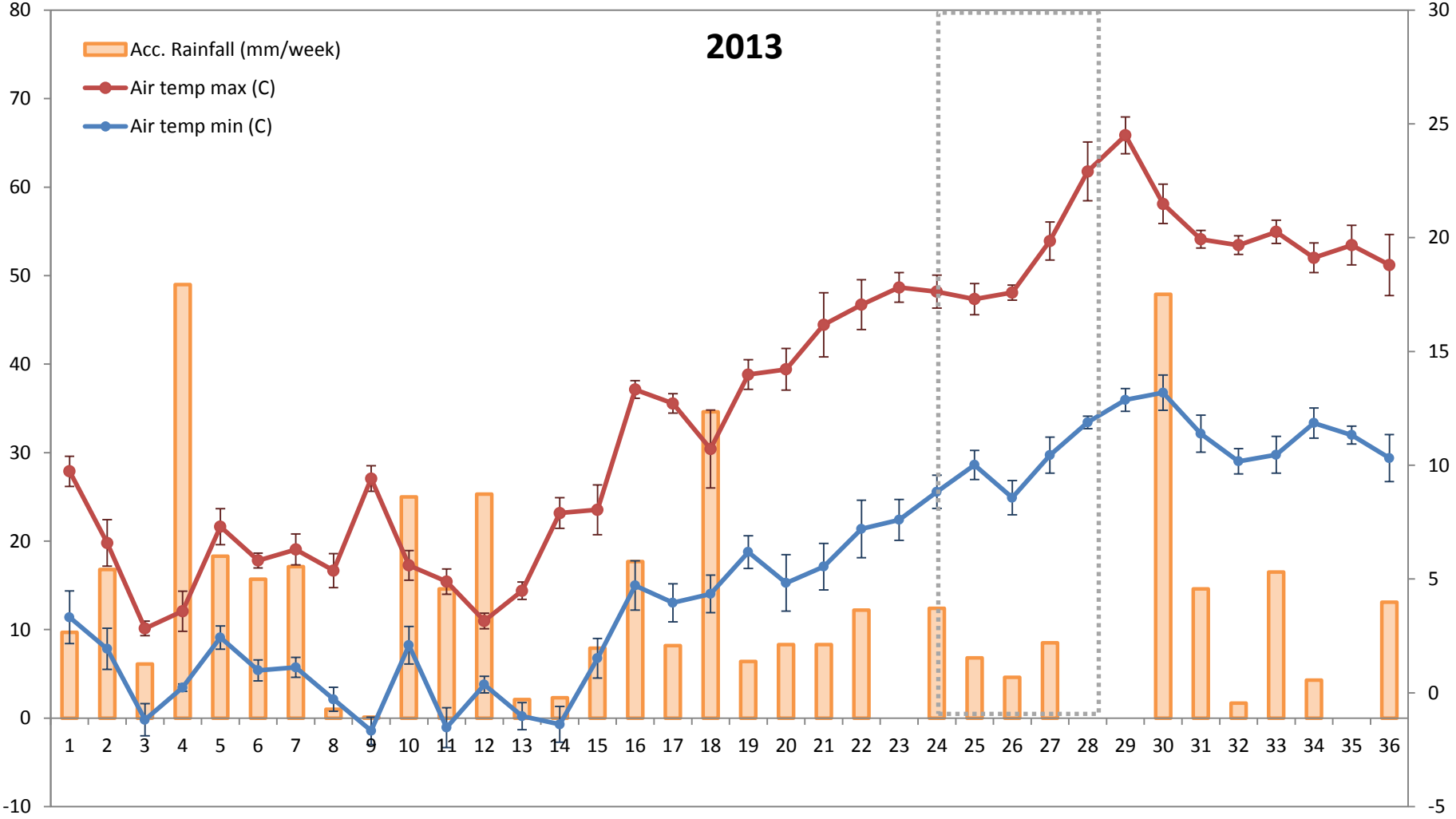
A) Script for analysing both years data together

```
VCOMPONENTS [FIXED=Year*Treatment*Genotype; FACTORIAL=32]
RANDOM=Year/Treatment/Rep/Col;\
 CONSTRAIN=pos
REML [PRINT=model,components,means,deviance,waldTests;
MAXCYCLE=15; FMETHOD=automatic;\
 PSE=differences; METHOD=AI] HI%
VMCOMP
[method=fplsd;DFMETHOD=tryfddf;dfgiven=49
0] Year
& Treatment
& Genotype
& Year.Treatment
& Year.Genotype
& Treatment.Genotype
& Year.Treatment.Genotype
```

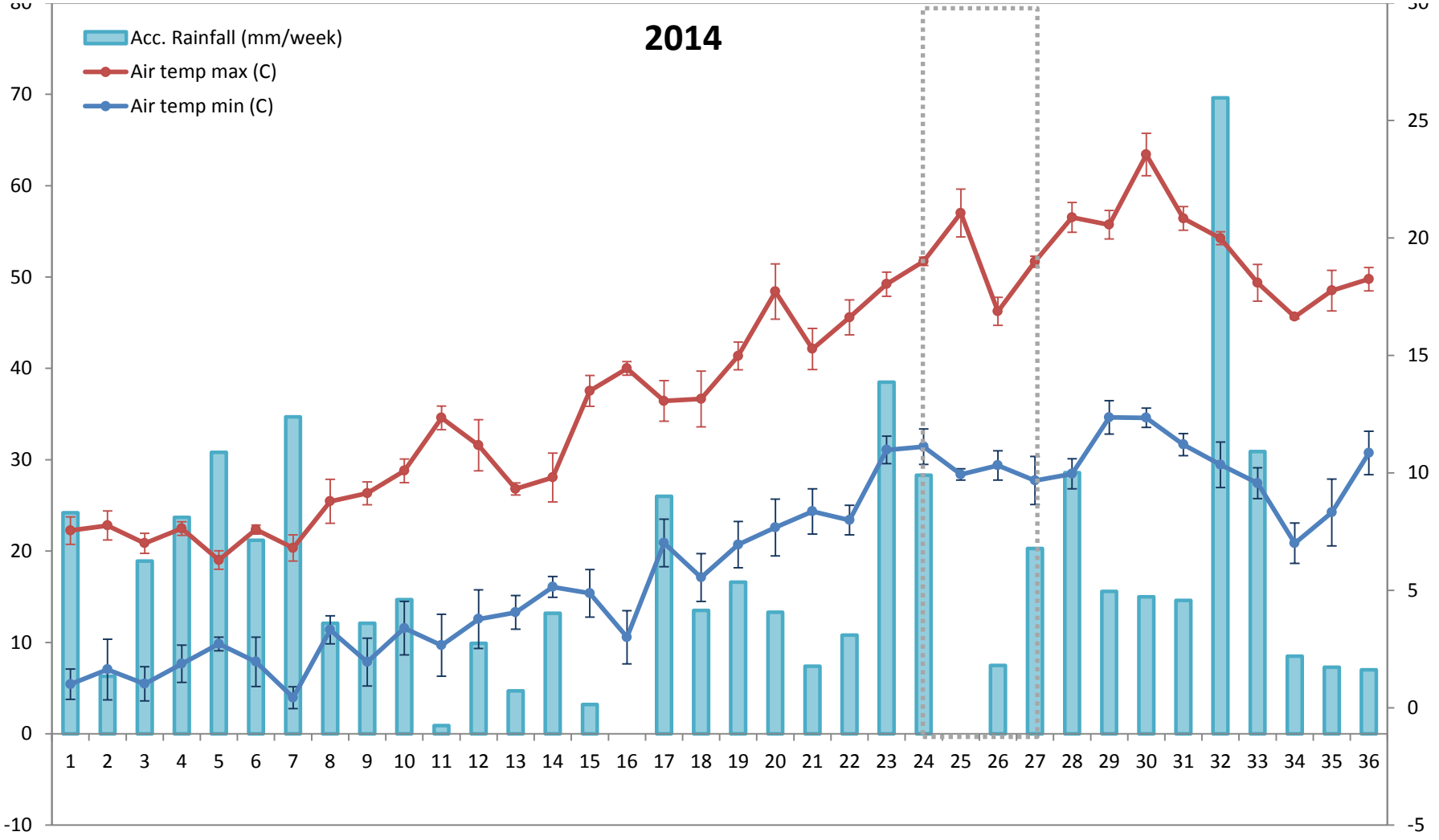
B) Script for analysing one year data

```
VCOMPONENTS [FIXED=Treatment*Genotype; FACTORIAL=32]
RANDOM=Treatment/Rep/Col;\
 CONSTRAIN=pos
REML [PRINT=model,components,means,deviance,waldTests;
MAXCYCLE=15; FMETHOD=automatic;\
 PSE=differences; METHOD=AI]
Heading_DAS
VMCOMP [method=fplsd;DFMETHOD=tryfddf;dfgiven=266] Treatment
& Genotype
& Treatment.Genotype
```

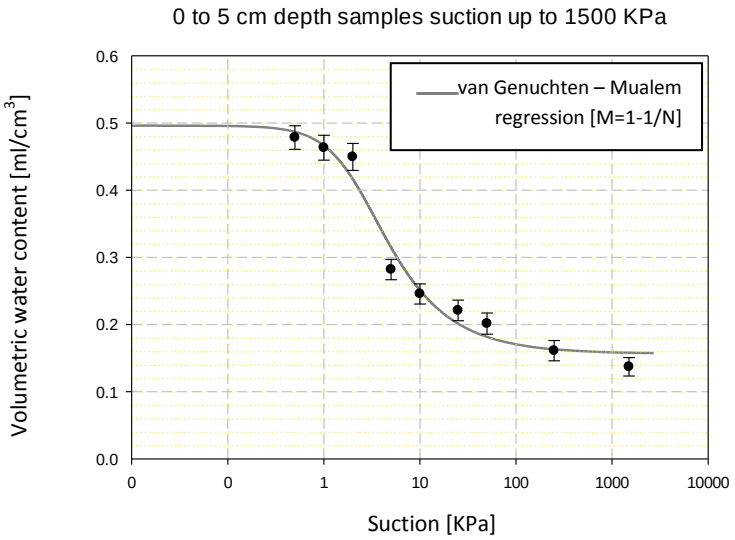
Appendix 3. Climate data before and throughout the field trial in (A) 2013 and (B) 2014. Accumulated rainfall (mm) values per week (x axes), air maximum and minimum average temperature values per week (± SE). Data obtained from James Hutton Institute weather station (56.45°N; 3.07°W). Field trial were established on the week 16 both years. Dotted square shows weeks when plants reached heading date.

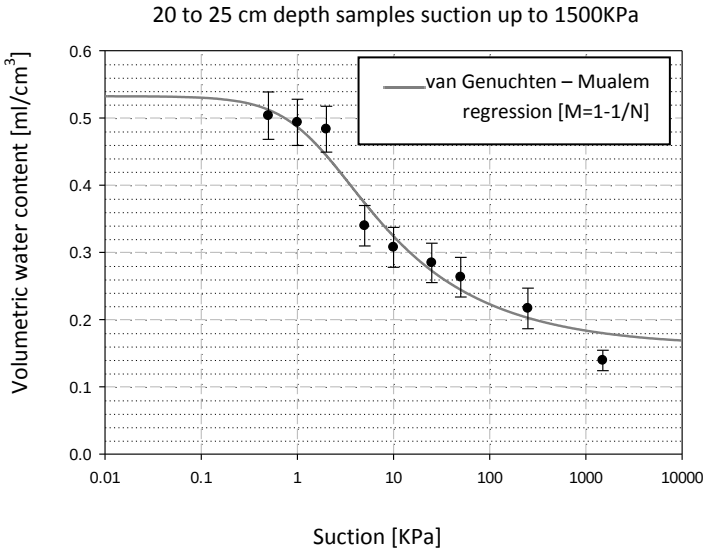


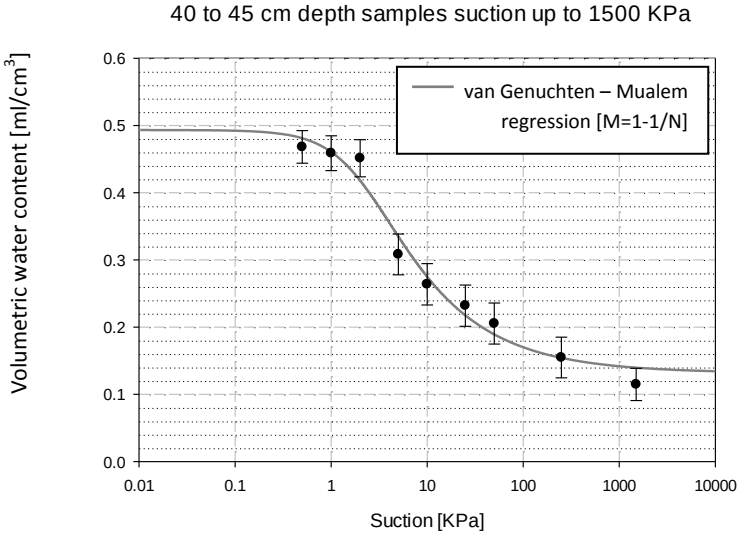
Appendix 3 (continued). Climate data before and throughout the field trial in (A) 2013 and (B) 2014 Accumulated rainfall (mm) values per week (x axes), air maximum and minimum average temperature values per week (± SE). Data obtained from James Hutton Institute weather station (56.45°N; 3.07°W). Field trial were established on the week 16 both years. Dotted square shows weeks when plants reached heading date.



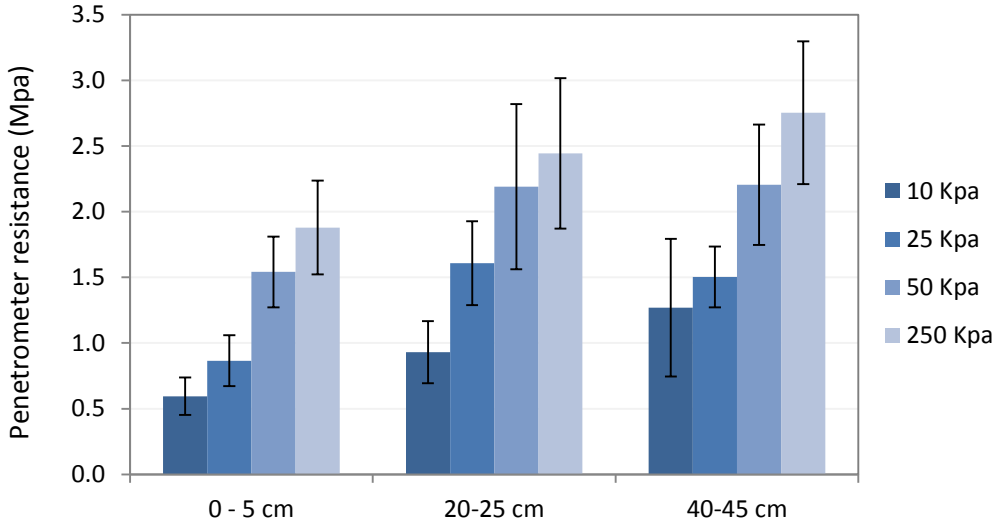
Appendix 4. Field soil water retention curves resulting from the mean van Genuchten parameters for eighteen field soil core sampled at three different depths at the field site in 2013.







Appendix 5. Penetrometer resistance (Mpa) for samples equilibrated at suctions 10 kPa, 25 kPa, 50 kPa and 250 kPa. Each bar corresponds to the mean value obtained for six cores sampled at each of the three different depths. Error bars indicate standard error of the mean



Appendix 6. Mixed model analysis for Dry yield and TGW considering each year separately.

Tests for fixed effects (Dry Yield 2013)

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	160.24	2	80.12	48.4	<0.001
Genotype	321.35	28	11.48	225.3	<0.001
Treatment.Genotype	99.35	56	1.77	226.4	0.002

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment.Genotype	99.35	56	1.77	226.4	0.002

Tests for fixed effects (Dry Yield 2014)

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	29.86	2	14.93	46.0	<0.001
Genotype	142.53	28	5.09	228.3	<0.001
Treatment.Genotype	74.92	56	1.34	228.7	0.072

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment.Genotype	74.92	56	1.34	228.7	0.072

Tests for fixed effects (TGW 2013)

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	23.86	2	11.93	8.9	0.003
Genotype	560.75	28	20.03	220.5	<0.001
Treatment.Genotype	119.32	56	2.13	221.4	<0.001

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment.Genotype	119.32	56	2.13	221.4	<0.001

Tests for fixed effects (TGW 2014)

Sequentially adding terms to fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment	51.47	2	25.74	9.0	<0.001
Genotype	408.47	28	14.59	221.1	<0.001
Treatment.Genotype	85.77	56	1.53	222.1	0.016

Dropping individual terms from full fixed model

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
Treatment.Genotype	85.77	56	1.53	222.1	0.016

Appendix 7- Genstat script for the marker–trait association analysis using a REML single-locus approach for 235 blocks of markers representing each loci.

```
"Load trait data from all_2013_2014_lines.gsh"

"Load marker data from iSelect_Genstat_CD.gsh - NB each offspring data
in columns pop[1...29]"

"put genotype identifiers into a text column geno_id"

text [nv=29] geno_id
for i=1...29
getattribute [att=identifier] newpop[i];save=att
text [nv=1] nn[i];val=att['identifier']
endfor
equate olds=nn;new=geno_id
print geno_id

"Transpose marker data so each SNP has a column snp[1] etc"

vequate newpop;snp
ftext Genotype;fGenotype

"merge data sets"

join [nindex=1; method=left] left=!p(fGenotype);\
right=!p(geno_id,snp[1...235]); new=!p(sgeno_id,ssnp[1...235])
group ssnp[1...235];fsnp[1...235]

"Run a loop including R2 and marker frequency count"
"loop starts here; run for each trait"

vari [nv=235]
prob_year,prob_treat,prob_snp,prob_yt,prob_ys,prob_ts,prob_yts
vari [nv=235]
SNP_mean1,SNP_mean2,SNP_mean2013_1,SNP_mean2013_2,SNP_mean2014_1,SNP_m
ean2014_2,\
SNP_meanD_1,SNP_meanD_2,SNP_meanI_1,SNP_meanI_2,SNP_meanM_1,SNP_meanM_
2,\
SNP_mean2013D_1,SNP_mean2013D_2,SNP_mean2014D_1,SNP_mean2014D_2,SNP_me
an2013I_1,SNP_mean2013I_2,\
SNP_mean2014I_1,SNP_mean2014I_2,SNP_mean2013M_1,SNP_mean2013M_2,SNP_me
an2014M_1,SNP_mean2014M_2,\
snpfreq,R2_G,R2_GT,R2_P,R2_PT

VCOMPONENTS [FIXED=Year*Treatment; FACTORIAL=32]
RANDOM=Genotype+Genotype.Treatment+\
Genotype.Year"+Genotype.Treatment.Year"+Year/Treatment/Rep/Col;\
 CONSTRAIN=pos
REML [PRINT=model,components,means,deviance,waldTests; MAXCYCLE=15;
FMETHOD=automatic;\
 PSE=differences; METHOD=AI] HI%
\vkeep Genotype+Genotype.Treatment ; comp=vcg1,vcgt1
vkeep Genotype+Genotype.Treatment+Genotype.Year ;
comp=vcg1,vcgt1,vcgy1

for i="36,42,49"1...235
tabu [pr=n,m;class=fsnp[i]] HI% ; nobs=tobs
vtable tobs ; vobs
calc vobs=vobs/24
\print vobs
calc snpfreq$[i]=vobs$[2]/(vobs$[1]+vobs$[2])
```

```
print
i,order$[i],marker$[i],Chromosome$[i],Position_cM$[i],snpfreq$[i]
VCOMPONENTS [FIXED=Year*Treatment*fsnp[i]; FACTORIAL=32]
RANDOM=Genotype+Genotype.Treatment+\
Genotype.Year"+Genotype.Treatment.Year"+Year/Treatment/Rep/Col;\
 CONSTRAIN=pos

REML [PRINT=model,components,means,deviance,waldTests; MAXCYCLE=15;
FMETHOD=automatic;\
 PSE=differences; METHOD=AI] HI%
vkeep [wmethod=add]
Year*Treatment*fsnp[i];fstat=ffstat[1...7];ndf=ndf[1...7];ddf=ddf[1...
7]
vkeep
terms=fsnp[i]+Year.fsnp[i]+Treatment.fsnp[i]+Year.Treatment.fsnp[i];\
means=tab[1...4];sed=sedmat[1...4]
vkeep Genotype+Genotype.Treatment+Genotype.Year ;
comp=vcg3,vcgt3,vcgy3
print tab[1...4]
print 'table 1'
vtable tab[1];vtab[1];class=classa
PRINT vtab[1]
calc SNP_mean1$[i]=vtab[1]$[1]
calc SNP_mean2$[i]=vtab[1]$[2]

print 'table 2'
vtable tab[2];vtab[2];class=class
unstack [data=class[2];idunstacked=class[1]]
vtab[2];unstacked=unstackedYS[1,2]
print unstackedYS[1,2]
calc SNP_mean2013_1$[i]=unstackedYS[1]$[1]
calc SNP_mean2013_2$[i]=unstackedYS[2]$[1]
calc SNP_mean2014_1$[i]=unstackedYS[1]$[2]
calc SNP_mean2014_2$[i]=unstackedYS[2]$[2]

print 'table 3'
vtable tab[3];vtab[3];class=classT
unstack [data=classT[2];idunstacked=classT[1]]
vtab[3];unstacked=unstackedTS[1,2]
print unstackedTS[1,2]
calc SNP_meanD_1$[i]=unstackedTS[1]$[1]
calc SNP_meanD_2$[i]=unstackedTS[2]$[1]
calc SNP_meanI_1$[i]=unstackedTS[1]$[2]
calc SNP_meanI_2$[i]=unstackedTS[2]$[2]
calc SNP_meanM_1$[i]=unstackedTS[1]$[3]
calc SNP_meanM_2$[i]=unstackedTS[2]$[3]

print 'table 4'
vtable tab[4];vtab[4];class=classYT
unstack [data=classYT[3];idunstacked=classYT[1,2]]
vtab[4];unstacked=unstackedYTS[1,2]
print unstackedYTS[1,2]
calc SNP_mean2013D_1$[i]=unstackedYTS[1]$[1]
calc SNP_mean2013D_2$[i]=unstackedYTS[2]$[1]
calc SNP_mean2014D_1$[i]=unstackedYTS[1]$[2]
calc SNP_mean2014D_2$[i]=unstackedYTS[2]$[2]
calc SNP_mean2013I_1$[i]=unstackedYTS[1]$[3]
calc SNP_mean2013I_2$[i]=unstackedYTS[2]$[3]
calc SNP_mean2014I_1$[i]=unstackedYTS[1]$[4]
calc SNP_mean2014I_2$[i]=unstackedYTS[2]$[4]
calc SNP_mean2013M_1$[i]=unstackedYTS[1]$[5]
calc SNP_mean2013M_2$[i]=unstackedYTS[2]$[5]
calc SNP_mean2014M_1$[i]=unstackedYTS[1]$[6]
```

```
calc SNP_mean2014M_2$[i]=unstackedYTS[2]$[6]


VCOMPONENTS [FIXED=Year*Treatment+fsnp[i]; FACTORIAL=32]
RANDOM=Genotype+Genotype.Treatment+\
Genotype.Year"+Genotype.Treatment.Year"+Year/Treatment/Rep/Col;\
 CONSTRAIN=pos
REML [PRINT=model,components,means,deviance,waldTests; MAXCYCLE=15;
FMETHOD=automatic;\
 PSE=differences; METHOD=AI] HI%

vkeep Genotype+Genotype.Treatment ; comp=vcg2,vcgt2

VCOMPONENTS [FIXED=Year*Treatment; FACTORIAL=32]
RANDOM=Genotype+Genotype.Treatment+\
Genotype.Year"+Genotype.Treatment.Year"+Year/Treatment/Rep/Col;\
 CONSTRAIN=pos
REML [PRINT=model,components,means,deviance,waldTests; MAXCYCLE=15;
FMETHOD=automatic;\
 PSE=differences; METHOD=AI] HI%
vkeep Genotype+Genotype.Treatment ; comp=vcg1,vcgt1

print vcg1,vcgt1,vcg2,vcgt2,vcg3,vcgt3,vcgy3

calc R2_G$[i]=100*(vcg1-vcg2)/vcg1
calc R2_GT$[i]=100*(vcgt1-vcgt3)/vcgt1
\print R2_G,R2_GT
\calc vv=vcg3+vcgt3+vcgy3
calc vv=vcg1+vcgt1+vcgy1
"I think this should use vcg1 etc instead"
print vv
calc R2_P$[i]=100*(vcg1-vcg2)/vv
calc R2_PT$[i]=100*(vcgt1-vcgt3)/vv
\print R2_P,R2_PT


print ffstat[]
CALC
prob_treat$[i],prob_year$[i],prob_snp$[i],prob_yt$[i],prob_ts$[i],prob
_ys$[i],prob_yts$[i] = \
CUF(ffstat[];ndf[];ddf[])
print
prob_year$[i],prob_treat$[i],prob_snp$[i],prob_yt$[i],prob_ys$[i],prob
_ts$[i],prob_yts$[i]
endfor


fspread
!(1...235),order,marker,Chromosome,Position_cM,snpfreq,R2_G,R2_GT,R2_P
,R2_PT,\
prob_year,prob_treat,prob_snp,prob_yt,prob_ys,prob_ts,prob_yts
fspreadsheet
!(1...235),order,marker,Chromosome,Position_cM,SNP_mean1,SNP_mean2,SNP
_mean2013_1,\
SNP_mean2013_2,SNP_mean2014_1,SNP_mean2014_2,\
SNP_meanD_1,SNP_meanD_2,SNP_meanI_1,SNP_meanI_2,SNP_meanM_1,SNP_meanM_
2,\
SNP_mean2013D_1,SNP_mean2013D_2,SNP_mean2014D_1,SNP_mean2014D_2,SNP_me
an2013I_1,SNP_mean2013I_2,\
SNP_mean2014I_1,SNP_mean2014I_2,SNP_mean2013M_1,SNP_mean2013M_2,SNP_me
an2014M_1,SNP_mean2014M_2
```

Appendix 8- Groups of polymorphic SNP markers on chromosome 1H and 5H removed due to ambiguous results. Markers[1] and genotypes arranged in columns and rows respectively

Marker[1]	11_21067	SCRI_RS_113745	12_30715	SCRI_RS_120053	SCRI_RS_120059	SCRI_RS_143952	SCRI_RS_214760
Chromosome	1H	1H	1H	1H	1H	5H	5H
Position (cM)	2.3	2.3	3.2	0.2	0.2	11	9.3
Caesarea	1	1	1	1	1	0	0
OSU012	1	1	1	1	1	0	0
OSU015	0	0	0	0	0	0	0
OSU018	0	0	0	0	0	0	0
OSU019	0	0	0	0	0	0	0
OSU024	1	1	1	0	0	0	0
OSU033	0	0	0	0	0	0	0
OSU035	0	0	0	0	0	0	0
OSU038	0	0	0	0	0	0	0
OSU040	0	0	0	0	0	0	0
OSU044	0	0	0	0	0	0	0
OSU047	1	1	1	1	1	0	0
OSU048	0	0	0	0	0	0	0
OSU051	0	0	0	0	0	0	0
OSU052	0	0	0	0	0	0	0
OSU053	0	0	0	0	0	0	0
OSU060	1	1	1	1	1	0	0
OSU061	0	0	0	0	0	0	0
OSU065	0	0	0	0	0	0	0
OSU074	1	1	1	1	1	0	0
OSU086	0	0	0	0	0	0	0
OSU090	1	1	1	1	1	0	0
OSU102	0	0	0	0	0	0	0
OSU105	0	0	0	0	0	0	0
OSU107	0	0	0	0	0	0	0
OSU124	0	0	0	0	0	0	0
OSU127	0	0	0	0	0	0	0
OSU137	0	0	0	0	0	0	0
OSU144	0	0	0	0	0	0	0
Harrington	1	1	1	1	1	1	1

[1] '0s' represent wild donor parent alleles (Caesarea 26-24)
'1s' represent elite recurrent parent alleles (cv. Harrington)

Appendix 9. (CD-ROM) RCLs genotypes determined for 1,848 SNP markers from the 9K SNP chip for barley (Comadran *et al.*, 2012)

Appendix 10- Wild barley chromosome regions introgressed on the elite barley genome per RCSL

		1H		**2H**		**3H**		**4H**		**5H**		**6H**		**7H**		***Total***	
RCSL	**Alleles**[1]	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	***%***	***cM***
OSU012	*Hv*	81.3	108.1	51.7	77.2	75.9	117.6	97	111.7	84.4	142.3	100	126.3	73.5	103.4	***79.6***	***786.6***
	Hsp	18.7	24.9	48.3	72.2	24.1	37.3	3	3.5	15.6	26.4	0	0	26.5	37.3	***20.4***	***201.6***
	?																
	No introg.	*2*		*1*		*1*		*1*		*2*				*2*		***9***	
OSU015	*Hv*	57.7	76.8	78.6	117.4	100	154.9	100	115.2	49	82.6	91.7	115.8	100	140.7	***81.3***	***803.4***
	Hsp	42.3	56.2	21.4	32	0	0	0	0	51	86.1	8.3	10.5	0	0	***18.7***	***184.8***
	?																
	No introg.	*1*		*1*						*2*		*1*				***5***	
OSU018	*Hv*	100	133	97.5	145.6	100	154.9	86.9	100.1	100	168.7	73.5	92.8	100	140.7	***94.7***	***935.8***
	Hsp	0	0	2.5	3.8	0	0	9.5	10.9	0	0	26.5	33.5	0	0	***4.9***	***48.2***
	?							3.6	4.2							***0.4***	***4.2***
	No introg.			*1*				*1*				*1*				***3***	
OSU019	*Hv*	78.7	104.7	82.5	123.2	100	154.9	100	115.2	73.3	123.7	54.9	69.4	100	140.7	***84.2***	***831.8***
	Hsp	21.3	28.3	17.5	26.2	0	0	0	0	22.8	38.5	45.1	56.9	0	0	***15.2***	***149.9***
	?									3.9	6.5					***0.7***	***6.5***
	No introg	*1*		*1*		*1*				*2*		*1*				***6***	
OSU024	*Hv*	45	59.9	90.8	135.7	78.1	120.9	83.9	96.6	99.1	167.2	97.7	123.4	52	73.2	***78.6***	***776.9***
	Hsp	55	73.1	8.6	12.8	21.8	33.8	15.5	17.8	0	0	0	0	48	67.5	***20.7***	***205***
	?			0.6	0.9	0.1	0.2	0.7	0.8	0.9	1.5	2.3	2.9			***0.6***	***6.3***
	No introg	*2*		*1*		*2*		*1*						*2*		***8***	
OSU033	*Hv*	83.4	110.9	92.8	138.6	54.5	84.4	97	111.7	97.3	164.1	39.7	50.2	46.1	64.9	***73.3***	***724.8***
	Hsp	16.6	22.1	7.2	10.8	45.5	70.5	2.9	3.3	2.7	4.6	60.3	76.1	53.9	75.8	***26.6***	***263.2***
	?							0.2	0.2							***0***	***0.2***
	No introg	*1*		*1*		*1*		*1*		*1*		*2*		*2*		***9***	

Appendix 10 (continued) Wild barley chromosome regions introgressed on the elite barley genome per RCSL.

		1H		2H		3H		4H		5H		6H		7H		*Total*	
RCSL	**Alleles[1]**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	***%***	***cM***
OSU035	*Hv*	94.7	126	100	149.4	100	154.9	70.9	81.7	72.1	121.6	84.5	106.7	73.8	103.8	***85.4***	***844.1***
	Hsp	5.3	7	0	0	0	0	29.1	33.5	27.9	47.1	15.5	19.6	26.2	36.9	***14.6***	***144.1***
	?																
	No introg	*1*				*1*		*1*		*1*		*1*		*1*		***6***	
OSU038	*Hv*	88.3	117.5	100	149.4	100	154.9	65.3	75.2	100	168.7	79	99.8	100	140.7	***91.7***	***906.2***
	Hsp	11.7	15.5	0	0	0	0	34.7	40	0	0	21	26.5	0	0	***8.3***	***82***
	?																
	No introg	*1*				*1*		*1*		*1*		*2*				***6***	
OSU040	*Hv*	100	133	100	149.4	66.9	103.6	78.9	90.9	69.3	116.9	92.6	117	100	140.7	***86.2***	***851.5***
	Hsp	0	0	0	0	26.9	41.7	21.1	24.3	20.6	34.8	7.2	9.1	0	0	***11.1***	***109.9***
	?					6.2	9.6			10.1	17	0.2	0.2			***2.7***	***26.8***
	No introg					*2*		*1*		*2*		*1*				***6***	
OSU044	*Hv*	69.3	92.2	99.2	148.2	97.2	150.5	100	115.2	52	87.7	62.3	78.7	97.8	137.6	***82***	***810.1***
	Hsp	30.7	40.8			0.5	0.7	0	0	48	81	37.7	47.6	2.2	3.1	***17.5***	***173.2***
	?			0.8	1.2	2.4	3.7									***0.5***	***4.9***
	No introg	*1*				*1*				*1*		*2*		*1*		***6***	
OSU047	*Hv*	90.2	120	81.7	122	100	154.9	76.5	88.1	89.4	150.9	91.2	115.2	100	140.7	***90.2***	***891.8***
	Hsp	3.8	5	16.7	24.9	0	0	23.5	27.1	9.4	15.9	7.2	9.1	0	0	***8.3***	***82***
	?	6	8	1.7	2.5					1.1	1.9	1.6	2			***1.5***	***14.4***
	No introg	*1*		*1*				*1*		*1*		*1*				***5***	
OSU048	*Hv*	61.6	81.9	91.8	137.1	78.3	121.3	82.3	94.8	100	168.7	90.1	113.8	77.8	109.5	***83.7***	***827.1***
	Hsp	38.4	51.1	8.2	12.3	21.7	33.6	17.7	20.4	0	0	8.4	10.6	10.3	14.5	***14.4***	***142.5***
	?											1.5	1.9	11.9	16.7	***1.9***	***18.6***
	No introg	*3*		*1*		*1*		*1*				*1*		*2*		***9***	

Appendix 10 (continued) Wild barley (*Hsp*) chromosome regions introgressed on the elite barley (*Hv*) genome per RCSL

		1H		2H		3H		4H		5H		6H		7H		***Total***	
RCSL	**Alleles[1]**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	***%***	***cM***
OSU051	*Hv*	94.7	126	78.6	117.4	76.4	118.4	76.5	88.1	100	168.7	79.4	100.3	86.4	121.6	***85.1***	***840.5***
	Hsp	5.3	7	21.4	32	23.6	36.5	23.5	27.1	0	0	20.6	26	13.6	19.1	***14.9***	***147.7***
	?																
	No introg	*1*		*2*		*2*		*2*				*2*		*1*		***10***	
OSU052	*Hv*	100	133	98.3	146.8	100	154.9	81.4	93.8	99	167	62.6	79.1	97.6	137.3	***92.3***	***911.9***
	Hsp	0	0	0.5	0.7	0	0	17.4	20	0	0	37.4	47.2	0	0	***6.9***	***67.9***
	?			1.3	1.9			1.2	1.4	1	1.7			2.4	3.4	***0.9***	***8.4***
	No introg			*1*				*1*				*1*				***3***	
OSU053	*Hv*	96.3	128.1	96.5	144.1	98	151.8	100	115.2	99.5	167.9	93.3	117.9	87.2	122.7	***95.9***	***947.7***
	Hsp	3.7	4.9	3.5	5.3	0	0	0	0	0.5	0.8	6.7	8.4	12.8	18	***3.7***	***36.6***
	?					2	3.1									***0.4***	***3.9***
	No introg	*1*		*1*								*1*		*2*		***5***	
OSU060	*Hv*	96.2	128	100	149.4	92.4	143.2	100	115.2	100	168.7	100	126.3	79	111.1	***95.3***	***941.9***
	Hsp	3.8	5	0	0	7.6	11.7	0	0	0	0	0	0	21	29.6	***4.7***	***46.3***
	?																
	No introg	*1*				*2*								*1*		***4***	
OSU061	*Hv*	100	133	86.3	128.9	78.9	122.2	82.7	95.3	83.3	140.6	100	126.3	61.5	86.5	***84.3***	***832.8***
	Hsp	0	0	13.4	20	21.1	32.7	17.3	19.9	16.7	28.1	0	0	35.4	49.8	***15.2***	***150.5***
	?			0.3	0.5									3.1	4.4	***0.5***	***4.9***
	No introg			*1*		*1*		*1*		*1*				*3*		***7***	
OSU065	*Hv*	100	133	68.7	102.6	86	133.2	69.4	79.9	69.9	118	34.2	43.2	100	140.7	***76***	***750.6***
	Hsp	0	0	30.5	45.6	14	21.7	30.6	35.3	30.1	50.7	65.8	83.1	0	0	***23.9***	***236.4***
	?			0.8	1.2											***0.1***	***1.2***
	No introg			*1*		*1*		*2*		*1*		*2*				***7***	

Appendix 10 (continued) Wild barley chromosome regions introgressed on the elite barley genome per RCSL

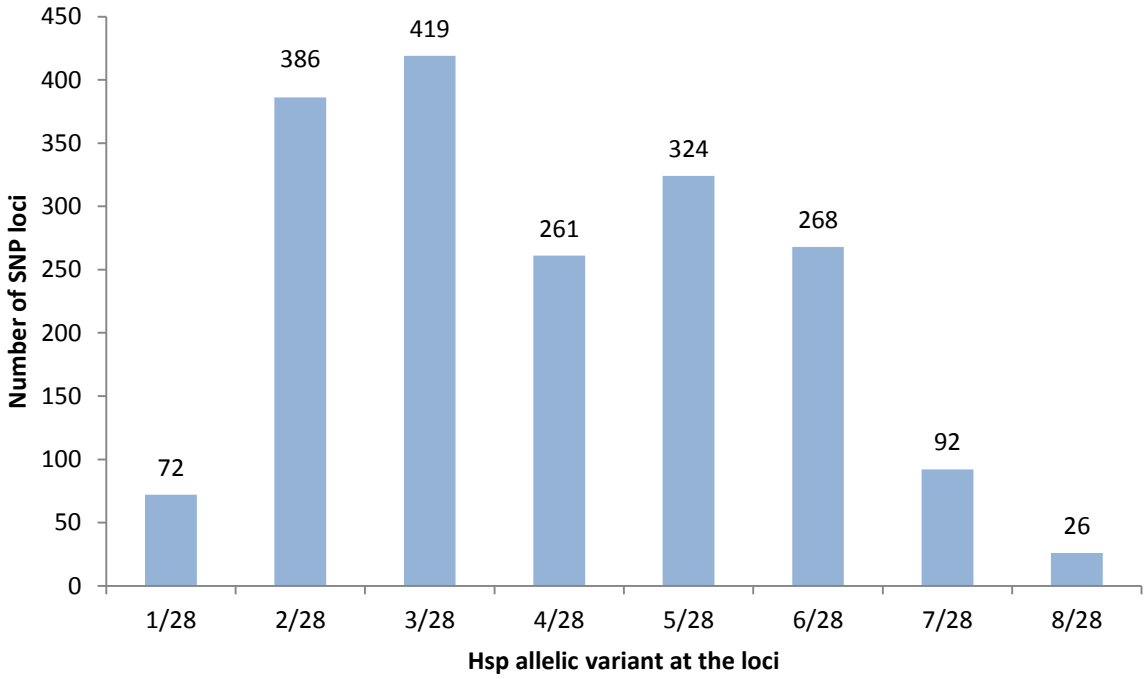
		1H		2H		3H		4H		5H		6H		7H		*Total*	
RCSL	**Alleles[1]**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	***%***	***cM***
OSU074	*Hv*	96.2	128	91.2	136.3	100	154.9	77.1	88.8	84.6	142.7	92.8	117.2	83.7	117.8	***89.6***	***885.7***
	Hsp	3.8	5	8.8	13.1	0	0	22.9	26.4	15.4	26	7.2	9.1	16.3	22.9	***10.4***	***102.5***
	?																
	No introg	*1*		*1*				*2*		*2*		*1*		*1*		***8***	
OSU086	*Hv*	76.3	101.5	83.9	125.3	66.9	103.6	66.2	76.3	100	168.7	88.7	112	100	140.7	***83.8***	***828.1***
	Hsp	23.7	31.5	16.1	24.1	33.1	51.3	33.8	38.9	0	0	11.3	14.3	0	0	***16.2***	***160.1***
	?																
	No introg	*1*		*3*		*1*		*1*				*1*				***7***	
OSU090	*Hv*	75.8	100.8	80.2	119.8	100	154.9	100	115.2	84.2	142	100	126.3	85.6	120.5	***89***	***879.5***
	Hsp	24.2	32.2	19.8	29.6	0	0	0	0	15.8	26.7	0	0	9.8	13.8	***10.4***	***102.3***
	?													4.5	6.4	***0.6***	***6.4***
	No introg	*1*		*1*						*2*				*2*		***6***	
OSU102	*Hv*	73.3	97.5	93.9	140.3	86.6	134.2	100	115.2	79.3	133.7	81.9	103.5	59.6	83.8	***81.8***	***808.2***
	Hsp	26.7	35.5	6.1	9.1	11.4	17.6	0	0	20.7	35	18.1	22.8	40.4	56.9	***17.9***	***176.9***
	?					2	3.1									***0.3***	***3.1***
	No introg	*3*		*1*		*1*				*2*		*1*		*2*		***10***	
OSU105	*Hv*	100	133	71.2	106.3	100	154.9	69.3	79.8	100	168.7	83.5	105.4	45.4	63.9	***82.1***	***811.5***
	Hsp	0	0	28	41.9	0	0	30.7	35.4	0	0	16.5	20.9	47.8	67.2	***16.7***	***165.4***
	?			0.8	1.2									6.8	9.6	***1.1***	***11.3***
	No introg			*1*				*2*				*2*		*2*		***7***	
OSU107	*Hv*	70.3	93.5	100	149.4	95	147.2	100	115.2	96.6	163	97.3	122.9	71	99.9	***90.2***	***891.1***
	Hsp	29.7	39.5	0	0	5	7.7	0	0	3.4	5.7	2.7	3.4	29	40.8	***9.8***	***97.1***
	?																
	No introg	*1*				*1*				*1*		*1*		*1*		***5***	

Appendix 10 (continued) Wild barley chromosome regions introgressed on the elite barley genome per RCSL

		1H		2H		3H		4H		5H		6H		7H		*Total*	
RCSL	**Alleles**[1]	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	**%**	**cM**	***%***	***cM***
OSU124	*Hv*	73.5	97.8	84.6	126.4	100	154.9	65.9	75.9	100	168.7	80.8	102.1	100	140.7	***87.7***	***866.5***
	Hsp	26.5	35.2	15.4	23	0	0	34.1	39.3	0	0	19.2	24.2	0	0	***12.3***	***121.7***
	?																
	No introg	*2*		*2*				*2*				*1*				***7***	
OSU127	*Hv*	79.4	105.6	94.5	141.2	100	154.9	100	115.2	100	168.7	100	126.3	52.6	74	***89.6***	***885.9***
	Hsp	20.6	27.4	5.5	8.2	0	0	0	0	0	0	0	0	47.4	66.7	***10.4***	***102.3***
	?																
	No introg	*2*		*1*										*1*		***4***	
OSU137	*Hv*	76.2	101.4	86.9	129.8	93.9	145.5	85.9	98.9	81.7	137.9	100	126.3	91.4	128.6	***87.9***	***868.4***
	Hsp	23.8	31.6	12.8	19.1	6	9.3	14.1	16.3	14.1	23.8	0	0	2.2	3.1	***10.4***	***103.2***
	?			0..3	0.5	0.1	0.1			4.1	7			6.4	9	***1.7***	***16.6***
	No introg	*1*		*1*		*1*		*1*		*1*				*1*		***6***	
OSU144	*Hv*	100	133	82.3	122.9	84.6	131	62.3	71.8	100	168.7	100	126.3	98	137.9	***90.2***	***891.6***
	Hsp	0	0	16.7	24.9	15.4	23.9	37.7	43.4	0	0	0	0	2	2.8	***9.6***	***95***
	?			1.1	1.6											***0.2***	***1.6***
	No introg			*1*		*1*		*2*						*1*		***5***	
Total introgressions per chromosome		*29*		*26*		*22*		*25*		*23*		*26*		*28*		***179***	

[1] *Hv: Hordeum vulgare* subsp. *vulgare*, i.e. cv. Harrington
Hsp: Hordeum vulgare subsp. *spontaneum*, i.e. Caesarea 26-24
? : Markers missing data
No. Introg: Number of exotic genome introgressions

Appendix 11. Minor allele frequency in the 28 RCSLs for 1848 SNP marker loci



Appendix 12- Set of markers selected for QTL analysis

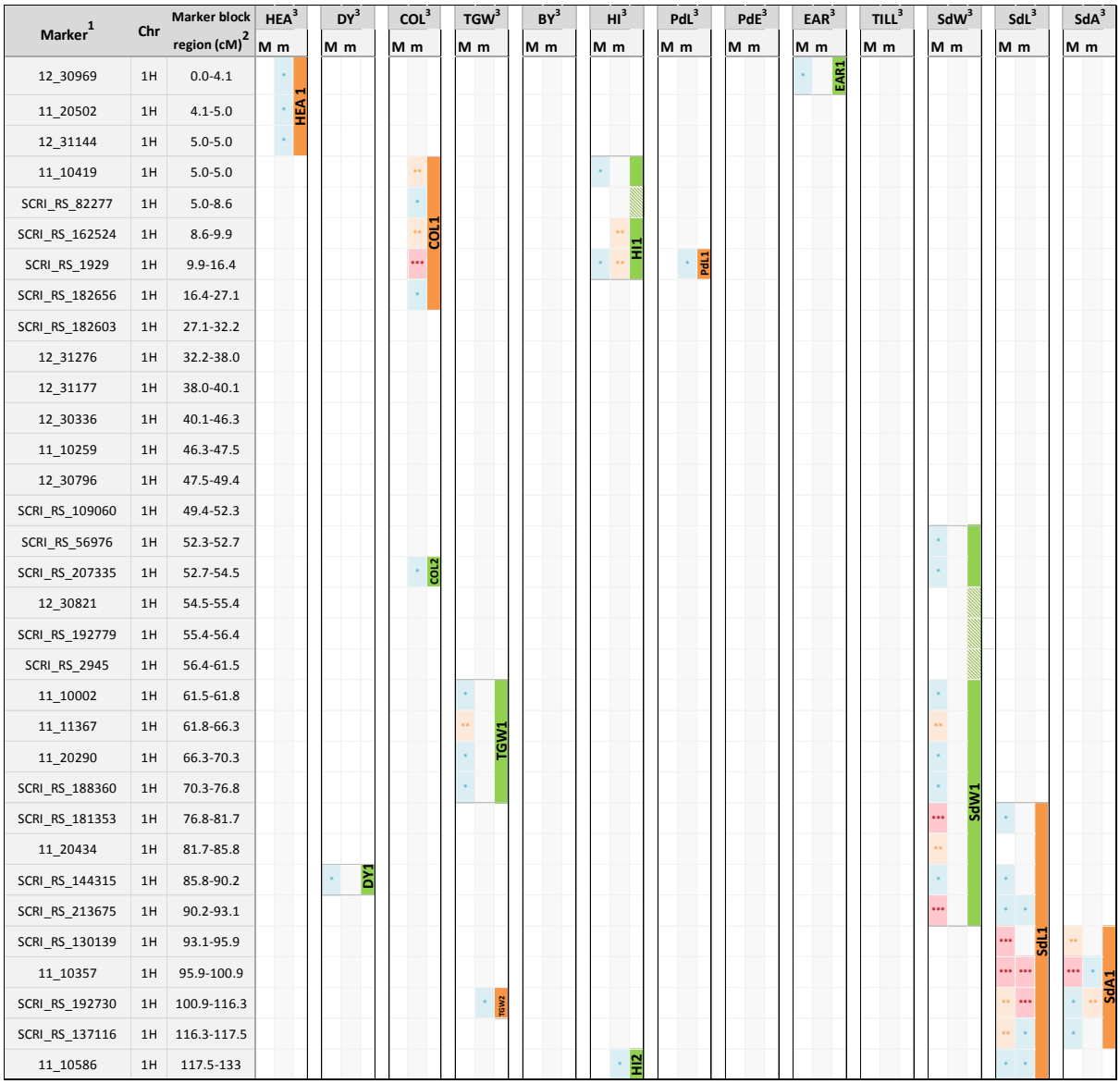
Chr[1]	No SNP blocks[2]	Av. block size (cM)[3]	Block range size (cM)[4]
1H	33	4.03	0.0-15.5
2H	39	3.83	0.0-20.4
3H	33	4.69	0.0-14.7
4H	28	4.76	0.0-14.7
5H	37	4.56	0.4-16.0
6H	30	4.21	0.0-13.7
7H	35	4.02	0.3-16.8
Total	*235*	*4.21*	*0.0-16.8*

[1] Chromosome
[2] Number of SNP blocks per chromosome
[3] Average size of the region represented by each block of SNP markers
[4] Maximum and minimum size of the region represented by the blocks of SNP markers

Appendix 13. (CD-ROM) Significant quantitative trait locus (QTLs) detected on the RCSLs exotic genetic library associated with thirteen morphological, developmental and agronomic traits.

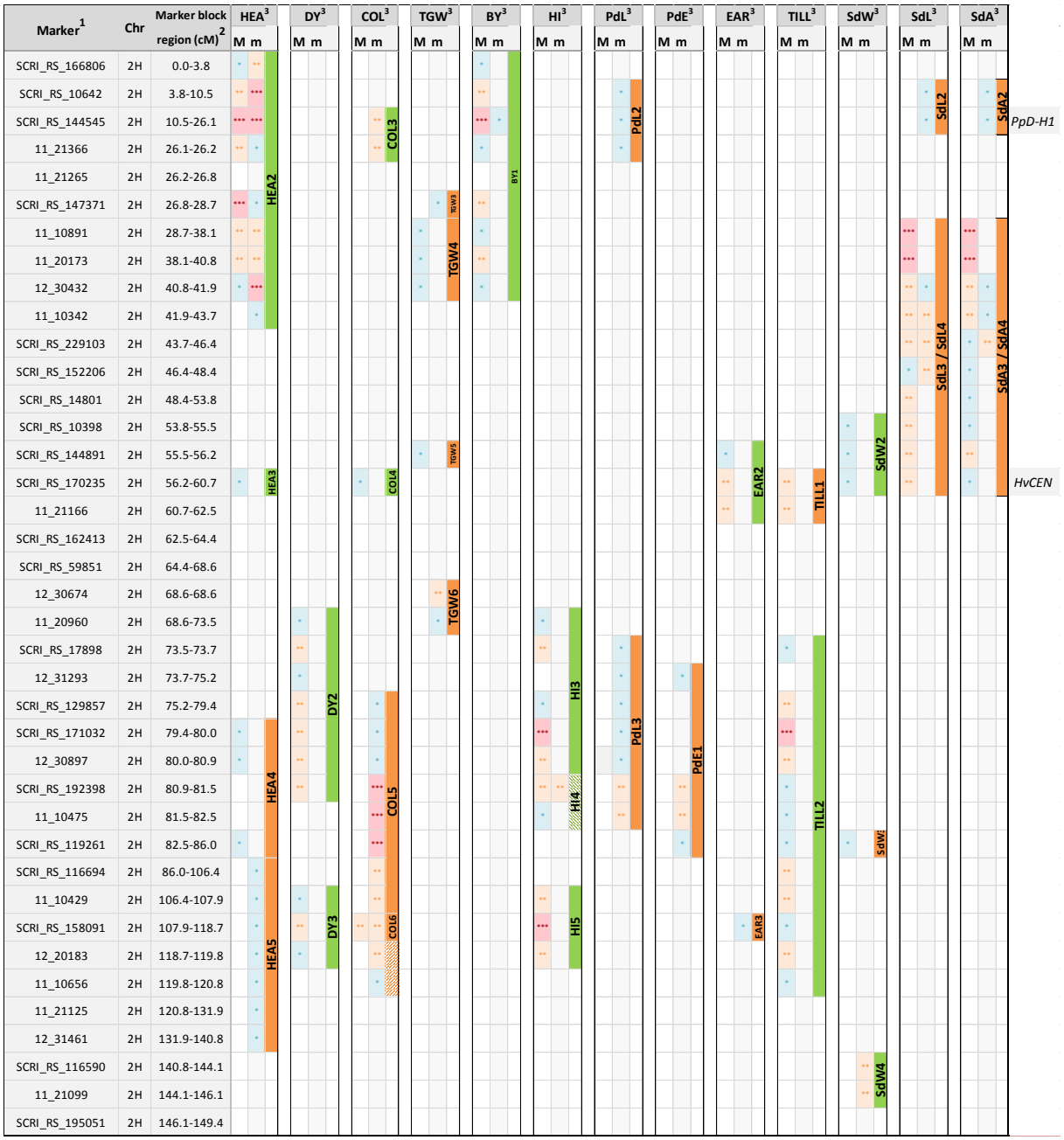
Appendix 14. Major and minor QTLs located in the REML single locus analysis for chromosomes A) 1H, B) 2H, C) 3H, D) 4H, E) 5H, F) 6H, and G) 7H.

A) Chromosome 1H



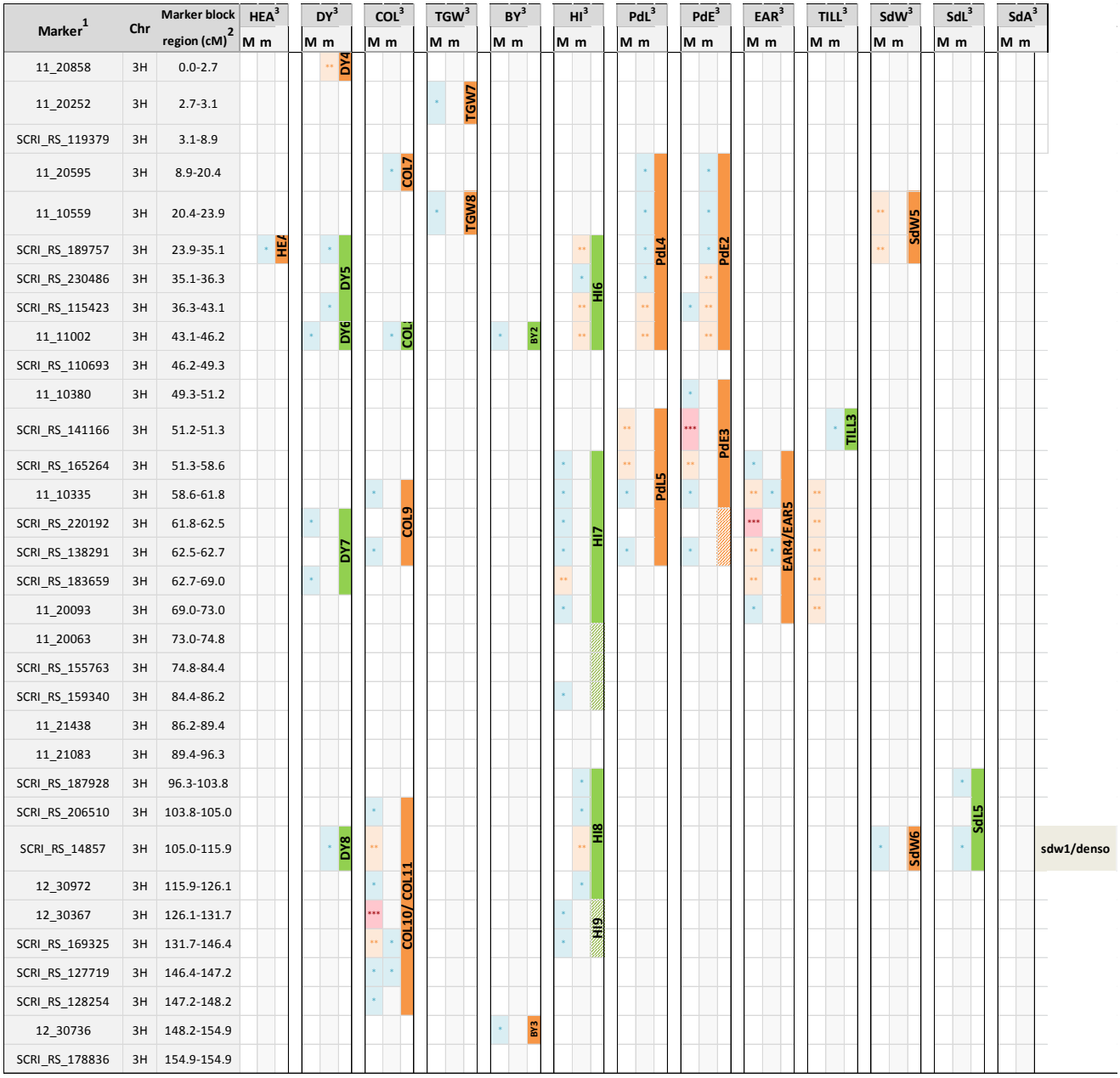
Appendix 14. Continued.

B) Chromosome 2H



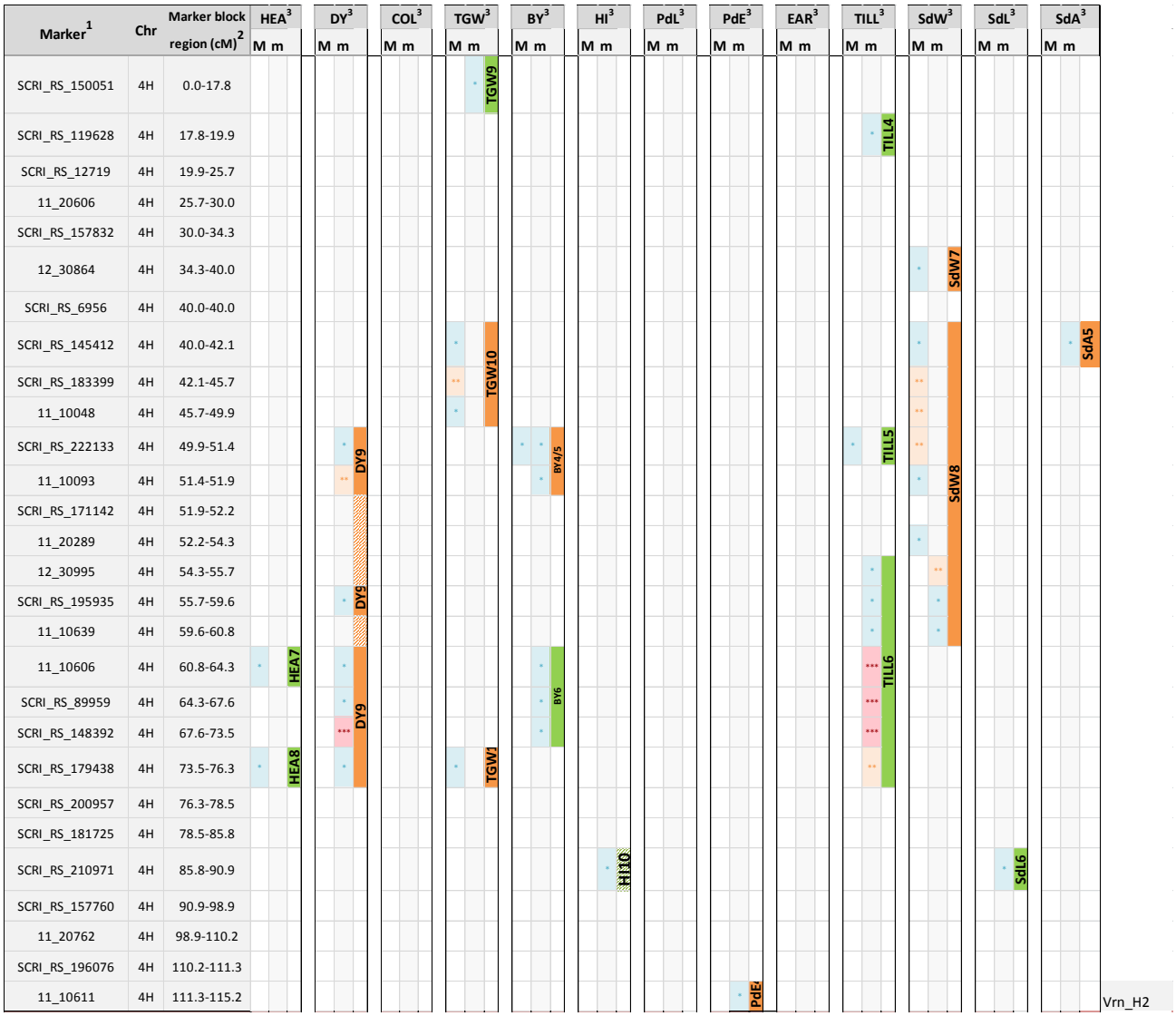
Appendix 14. Continued.

C) Chromosome 3H



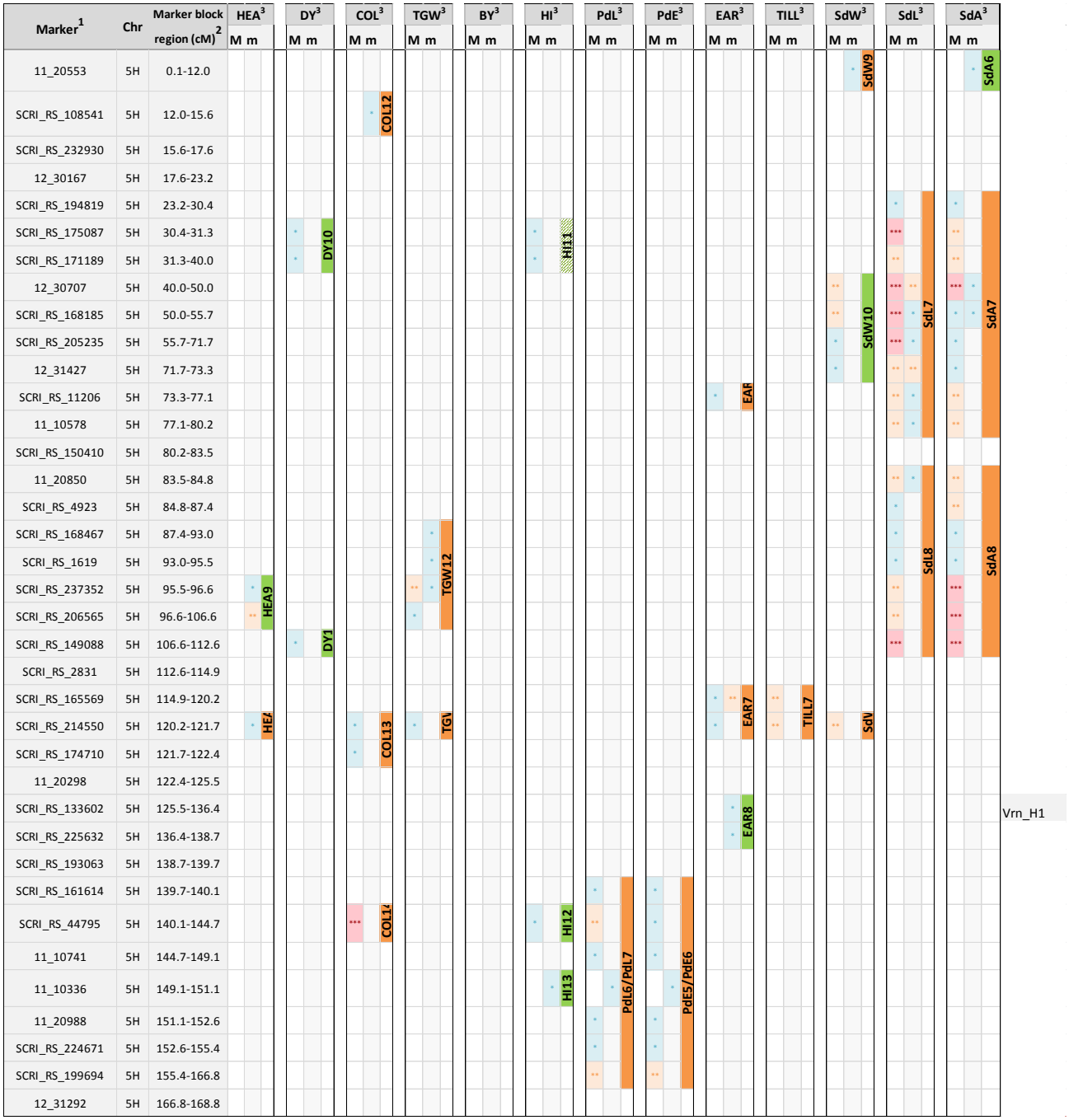
Appendix 14. Continued.

D) Chromosome 4H



Appendix 14. Continued.

E) Chromosome 5H



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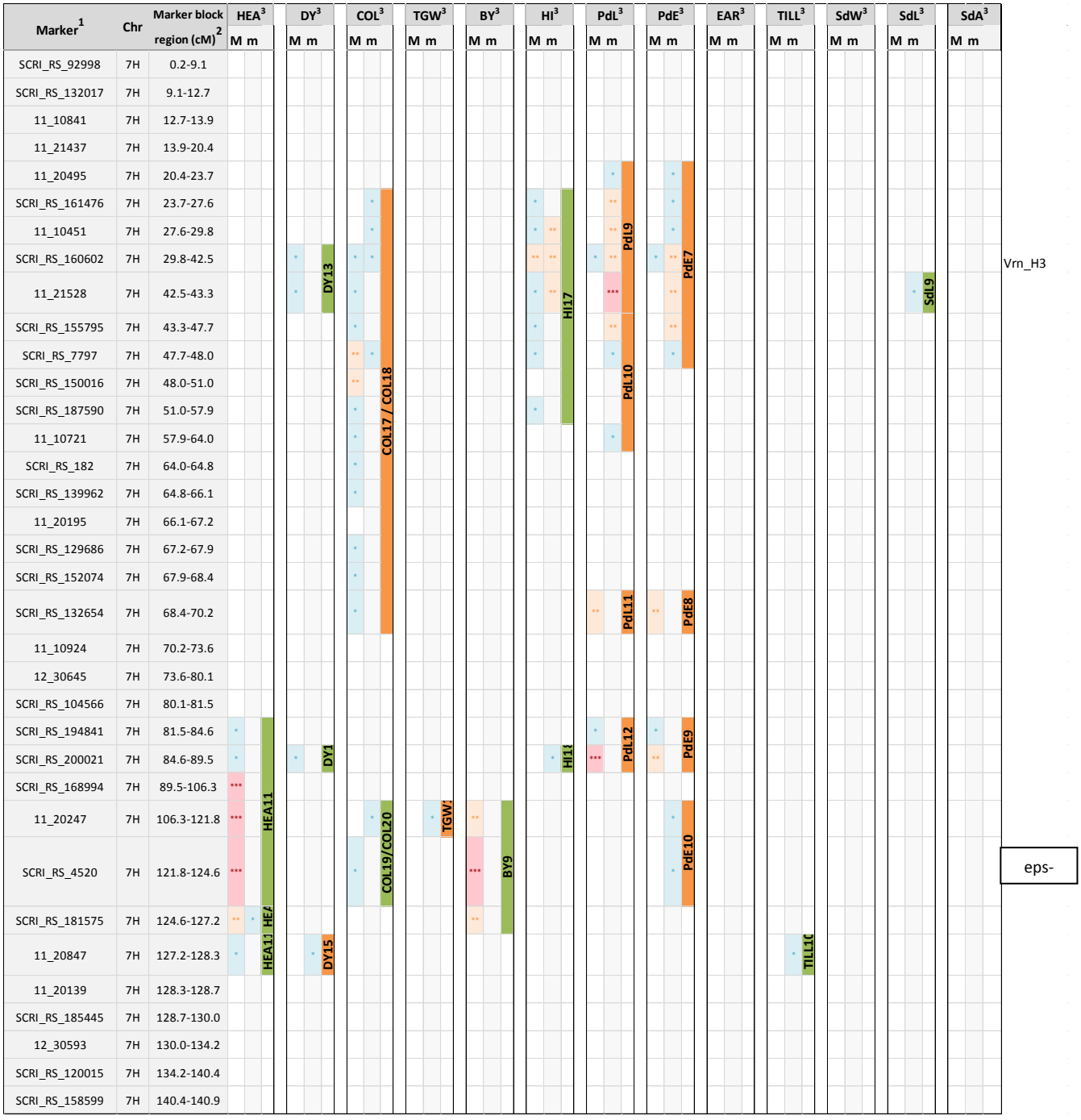
Appendix 14. Continued.

F) Chromosome 6H

Marker[1]	Chr	Marker block region (cM)[2]	HEA[3]	DY[3]	COL[3]	TGW[3]	BY[3]	HI[3]	PdL[3]	PdE[3]	EAR[3]	TILL[3]	SdW[3]	SdL[3]	SdA[3]
			M m	M m	M m	M m	M m	M m	M m	M m	M m	M m	M m	M m	M m
SCRI_RS_168487	6H	0.3-5.2			* COL15			* HI14	* PdL8						
SCRI_RS_153023	6H	5.2-6.4													
SCRI_RS_211856	6H	6.4-10.8													
SCRI_RS_206183	6H	10.8-24.5													
SCRI_RS_138556	6H	24.5-32.2													
11_10994	6H	32.2-33.2													
11_10799	6H	33.2-44.1													
11_20936	6H	44.1-46.5													
SCRI_RS_213547	6H	46.5-47.5													
SCRI_RS_155654	6H	47.5-47.9									* ** EAR9/ EAR10				
SCRI_RS_162581	6H	47.9-48.8									*				
11_20743	6H	48.8-50.2									* *				
SCRI_RS_133544	6H	50.2-50.2									** **				
12_30133	6H	50.2-52.9									** **				
11_21473	6H	52.9-54.9						*			**				
11_21124	6H	54.9-57.2		* DY12				** HI15			**	* TILL8/TIL9			
SCRI_RS_153707	6H	57.2-62.7		* DY12	*			** HI15			**	** TILL8/TIL9			
11_11483	6H	62.7-64.3		* DY12	COL16						**	TILL8/TIL9			
SCRI_RS_115440	6H	64.3-65.7		DY12	COL16		** BY7				*	TILL8/TIL9			
SCRI_RS_149556	6H	65.7-69.3		DY12	COL16		BY7				* **	* TILL8/TIL9			
SCRI_RS_225193	6H	69.3-72.9		* DY12	* COL16	* TGW14	BY7	* HI16			** ***	* TILL8/TIL9			
SCRI_RS_228493	6H	72.9-85.7			** COL16	TGW14	BY7	** HI16			** **	* TILL8/TIL9			
SCRI_RS_165322	6H	85.7-86.3			*** * COL16	TGW14	** BY8	** HI16			* ***	* TILL8/TIL9			
SCRI_RS_164037	6H	86.3-93.1			COL16	TGW14	BY8	HI16			* **	** TILL8/TIL9			
SCRI_RS_124224	6H	93.1-100.4			COL16	TGW14	* BY8	HI16			EAR9/ EAR10	* TILL8/TIL9	* SdW12		
SCRI_RS_8034	6H	100.4-103.8			COL16	TGW14	BY8	HI16			EAR9/ EAR10	TILL8/TIL9	* SdW12		
SCRI_RS_169022	6H	103.8-115.9			COL16	TGW14	BY8	HI16			EAR9/ EAR10	TILL8/TIL9	SdW12		
SCRI_RS_138188	6H	115.9-117.5			COL16	TGW14	BY8	HI16			EAR9/ EAR10	TILL8/TIL9	SdW12		
SCRI_RS_151280	6H	117.5-126.6			COL16	** TGW15	BY8	HI16			EAR9/ EAR10	TILL8/TIL9	SdW12		
12_30956	6H	126.6				TGW15									

Appendix 14. Continued.

G) Chromosome 7H



1. Marker representing each of the 235 blocks of markers
2. Marker block region established by the genetic position corresponding to the first SNP markers defining adjacent loci
3. Marker-trait associations established at the marker main effect level (M) and in the interaction with the treatment (m) for thirteen quantitative traits (HEA: heading date, DY: dry yield, COL: collar height, TGW: thousand grain weight, BY: biomass yield, HI: harvest index, PdL: peduncle length, PdE: peduncle extrusion, EAR: ear length, TILL: number of tillers, SdW: seed width, SdL: seed length, SdA: seed area). The exotic alleles at the loci contributed to reduce (QTLs in green) or increase (QTLs in orange) significantly the estimated mean value for the trait compared to the effect of the recurrent parent alleles on the phenotype. Each QTL spans across adjacent blocks of markers showing significant effects in the same direction. The level of significance for each block of markers is indicated with ***P<0.001(red),**P<0.01(orange),*P<0.05 (blue), blanks: not significant association

Appendix 15- Thirty-seven QTLs targeted to select potential candidate genes using the barley new genome assembly.

		Genetic map position (iSelect)[3]			Physical position[4]		**Annotated genes[5]**
QTL[1]	Flanking markers[2]	Chr	Position (cM)	Distance (cM)	Position (bp)	Distance (Mbp)	
HEA2	SCRI_RS_10642	2H	3.8	36.3	5023280	46.99	**914**
	11_10919	2H	40.1		52013910		
HEA3	SCRI_RS_170235	2H	56.2	3.7	239094200	330.80	**1352**
	SCRI_RS_165574	2H	59.9		569894938		
HEA4	SCRI_RS_171032	2H	79.4	3.4	650676630	13.51	**133**
	11_21037	2H	82.8		664191557		
HEA7	SCRI_RS_166817	4H	61.4	2	559888997	7.48	**68**
	SCRI_RS_139806	4H	63.4		567371272		
HEA8	SCRI_RS_179438	4H	73.5	0.2	586059954	0.44	**15**
	SCRI_RS_155536	4H	73.7		586499258		
HEA11	SCRI_RS_200021	7H	84.6	42.6	573625202	66.17	**953**
	11_20847	7H	127.2		639792929		
COL4	SCRI_RS_170235	2H	56.2	3.7	239094200	330.80	**1352**
	SCRI_RS_165574	2H	59.9		569894938		
COL6	11_10538	2H	109.4	5.5	707452504	6.33	**152**
	SCRI_RS_179555	2H	114.9		713777779		
COL9	SCRI_RS_230975	3H	59	3.5	517643330	22.86	**198**
	SCRI_RS_138291	3H	62.5		540504713		
COL10	SCRI_RS_206510	3H	103.8	43.4	625621479	60.76	**1137**
	SCRI_RS_128254	3H	147.2		686381102		
COL13	SCRI_RS_214550	5H	120.2	2.2	590540389	4.43	**76**
	11_20298	5H	122.4		594971889		
COL14	SCRI_RS_44795	5H	140.1	4.6	624404370	6.77	**136**
	SCRI_RS_14527	5H	144.7		631177934		
COL16	SCRI_RS_114613	6H	57.2	28.5	394381749	147.45	**1114**
	SCRI_RS_165322	6H	85.7		541832053		
COL18	SCRI_RS_160602	7H	29.8	38.6	34820994	107.57	**1123**
	SCRI_RS_171786	7H	68.4		142388927		
COL20	11_10797	7H	124.1	0	635305121	0.07	**4**
	SCRI_RS_193330	7H	124.1		635236035		
TGW1	11_21431*	1H	59.4	17.4	453000119	35.82	**395**
	SCRI_RS_181353	1H	76.8		488815440		
TGW4	SCRI_RS_7026	2H	26.2	13.9	34878992	17.13	**213**
	11_10919	2H	40.1		52013910		
TGW5	SCRI_RS_144891	2H	55.5	0.4	174057756	50.95	**270**
	SCRI_RS_198865	2H	55.9		225004115		
TGW7	11_20252	3H	2.7	0.4	3868737	0.31	**18**
	SCRI_RS_180343	3H	3.1		4180557		
TGW8	11_10559	3H	20.4	0.4	23094230	0.10	**7**
	12_30920	3H	20.8		23190018		
TGW10	SCRI_RS_183399	4H	42.1	3.6	30937151	5.79	**71**
	11_10048	4H	45.7		36724174		

Appendix 15. Continued

QTL[1]	Flanking markers[2]	Genetic map position (iSelect)[3]			Physical position[4]		Annotated genes[5]
		Chr	Position (cM)	Distance (cM)	Position (bp)	Distance (Mbp)	
TGW11	SCRI_RS_179438	4H	73.5	2.8	586059954	4.35	**56**
	SCRI_RS_200957 *	4H	76.3		590413554		
TGW12	SCRI_RS_237352	5H	95.5	3.4	561601110	4.35	**82**
	12_30456	5H	98.9		565946580		
TGW13	SCRI_RS_214550	5H	120.2	1.5	590540389	2.98	**51**
	SCRI_RS_174710	5H	121.7		593523876		
SdW1	SCRI_RS_56976	1H	52.3	6.8	406534901	46.26	**390**
	SCRI_RS_194015	1H	59.1		452790401		
SdW2	SCRI_RS_10398	2H	53.8	6.1	113049465	456.85	**2084**
	SCRI_RS_165574	2H	59.9		569894938		
SdW3	SCRI_RS_119261	2H	82.5	0.3	663531310	0.66	**13**
	11_21037	2H	82.8		664191557		
SdW5	11_10559	3H	20.4	5.1	23094230	3.59	**92**
	SCRI_RS_222975	3H	25.5		26687478		
SdW6	11_11330	3H	105.3	4.5	628031246	6.90	**122**
	12_31238	3H	109.8		634931050		
SdW7	12_30864	4H	34.3	19.7	23604187	452.33	**2001**
	SCRI_RS_208828	4H	54		475931094		
SdW10	12_30707	5H	40	36.2	31296302	504.51	**2919**
	SCRI_RS_235416	5H	76.2		535809104		
SdW11	SCRI_RS_214550	5H	120.2	1.5	590540389	2.98	**51**
	SCRI_RS_174710	5H	121.7		593523876		
SdL1	SCRI_RS_181353	1H	76.8	56.2	488815440	69.21	**452**
	11_10443	1H	133		558025165		
SdL3	11_11073	2H	39.4	20.5	50233938	519.66	**2713**
	SCRI_RS_165574	2H	59.9		569894938		
SdL7	SCRI_RS_85089	5H	23.6	56.9	12381405	529.48	**3251**
	SCRI_RS_239097	5H	80.5		541861278		
SdL8	SCRI_RS_4923	5H	84.8	24.9	548710107	28.49	**572**
	SCRI_RS_231239	5H	109.7		577199505		
GLS1	12_30969	1H	0	3.2	977855	0.58	**20**
	12_30715	1H	3.2		1562067		

[1] Major QTLs selected to identify the number of potential candidate genes according to the new assembly
[2] iSelect markers with genetic and physical map position selected to define each QTL
[3] Markers genetic map position and distance according to Comadran *et at.* (2012)
[4] Markers physical position and distance according to the barley new genome assembly (IBSC, 2016)
[5] Number of high-confidence annotated genes for the targeted region

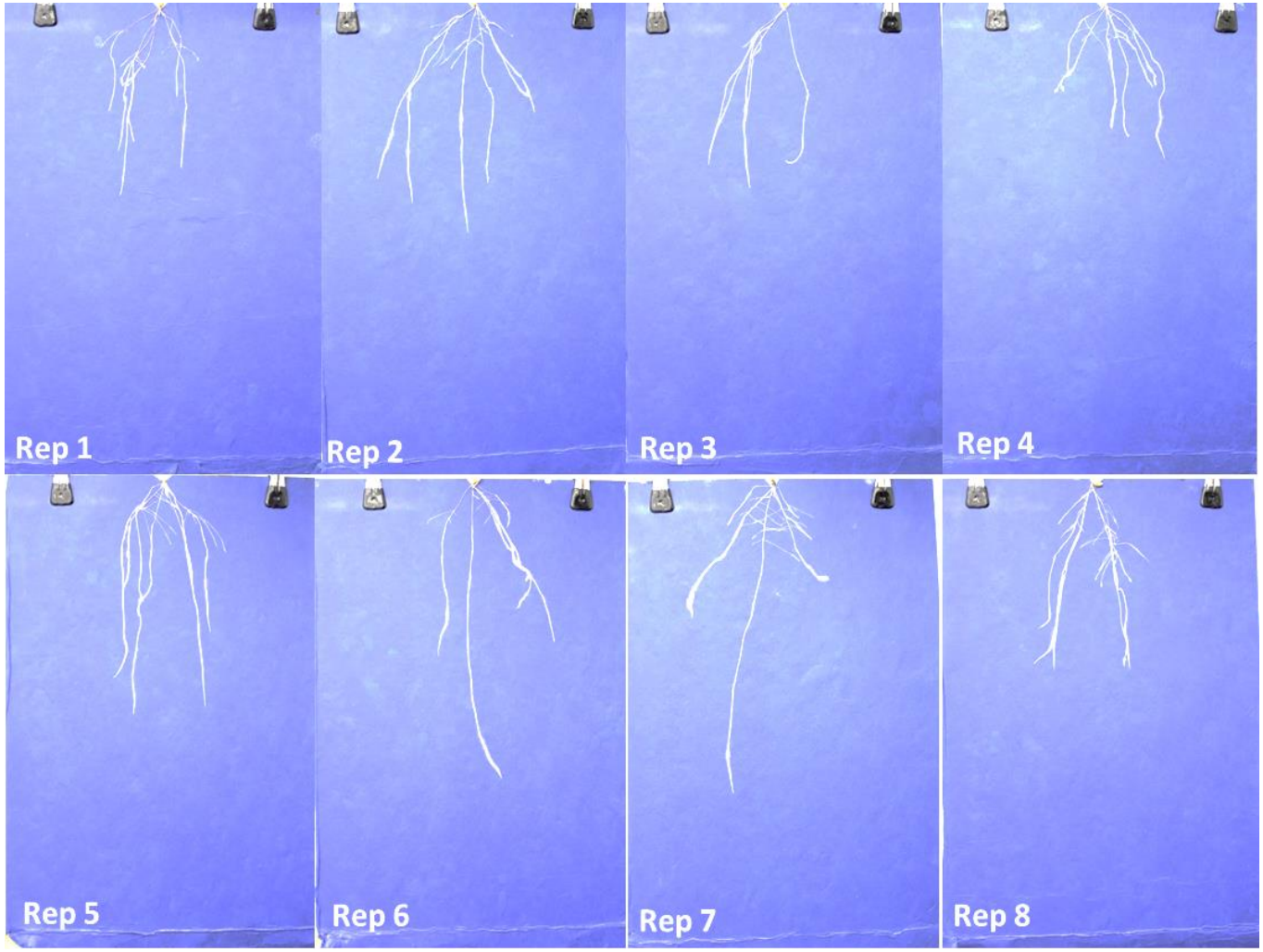
Appendix 16.CD_ROM High confidence genes found in the target region shared across SdW11 and TGW13 on 5H (120.2cM) using (A) BARLEYMAP based on the IBSC (2012) assembly and (B) on the new genome assembly (IBSC, 2016)

Appendix 17. CD-ROM. High confidence genes found in the target region shared across SdW5 and TGW8 on 3H (20.4-25.5 cM) using (A) BARLEYMAP based on the IBSC (2012) assembly and (B) on the new genome assembly (IBSC, 2016)

Appendix 18. CD-ROM. High confidence genes found in the target GLS1 on 1H (0 to 3.2 cM) using (A) BARLEYMAP based on the IBSC (2012) assembly and (B) on the new genome assembly (IBSC, 2016)

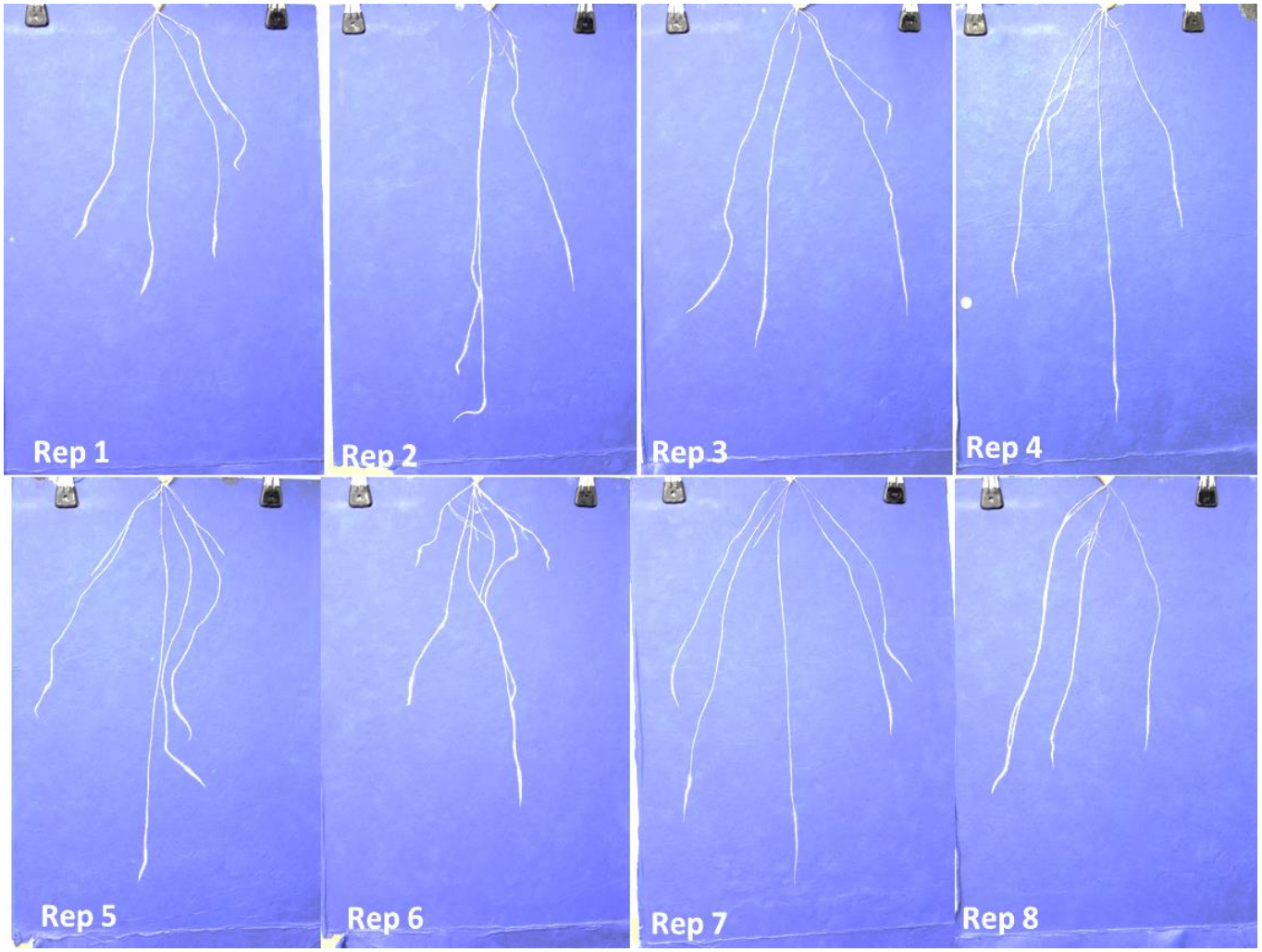
Appendix 19. Last time-point image after sixteen days of growth in 2D pouches for the eight replicates of A) OSU048, B) OSU044, C) cv. Harrington, D) OSU060, E) OSU052, F) OSU144

A) OSU048



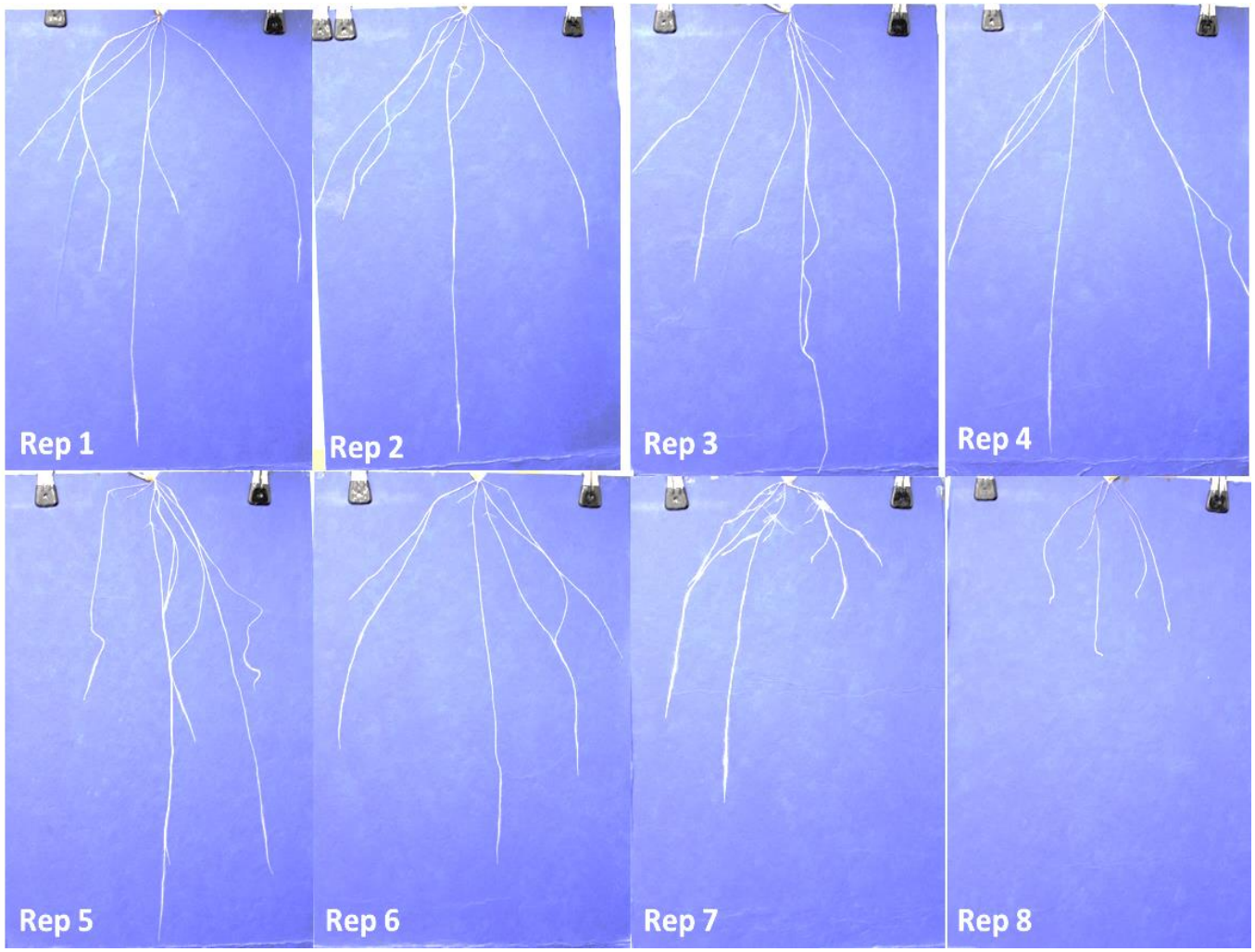
Appendix 19. Continued

B) OSU044



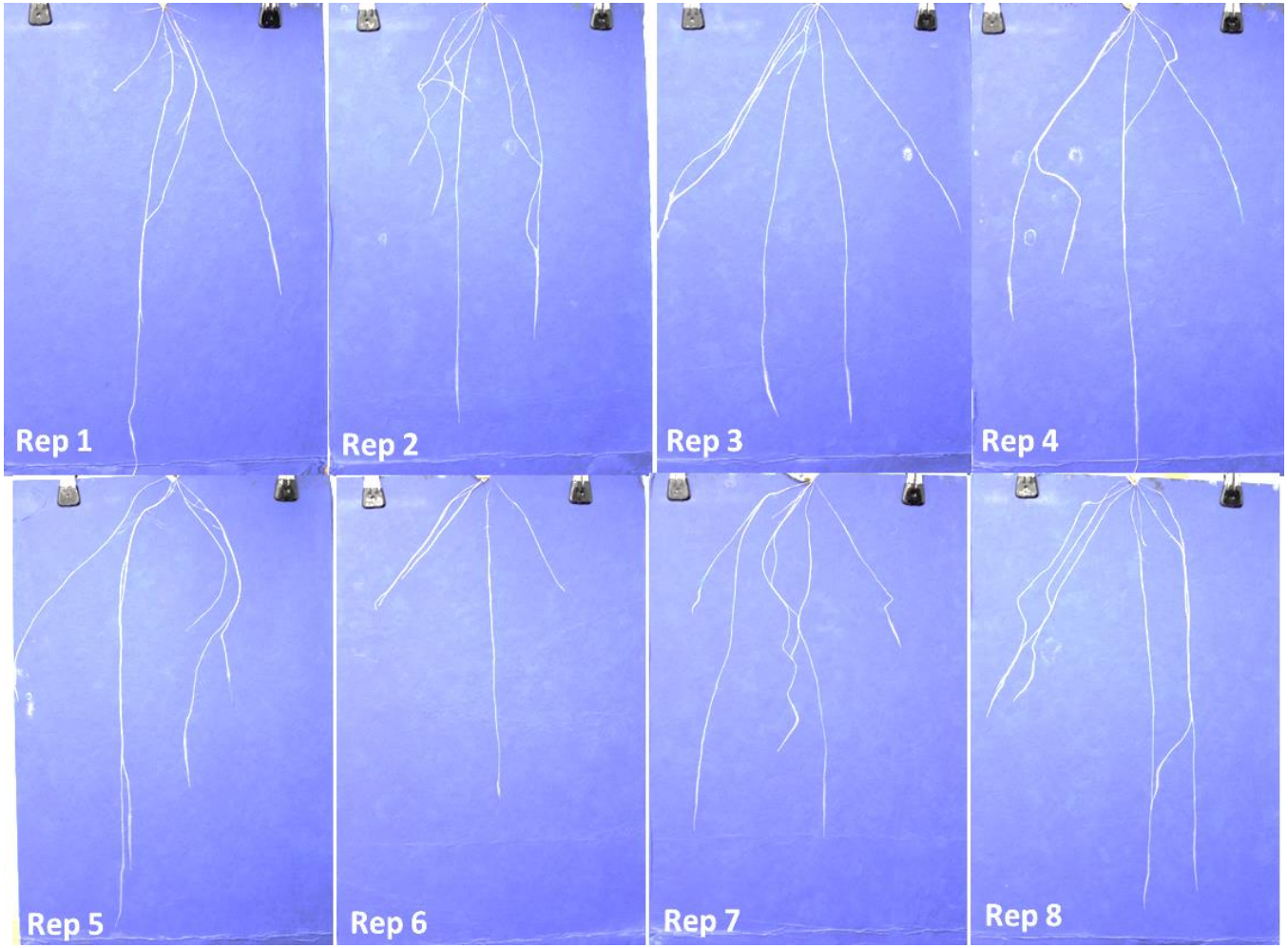
Appendix 19. Continued

C) cv. Harrington



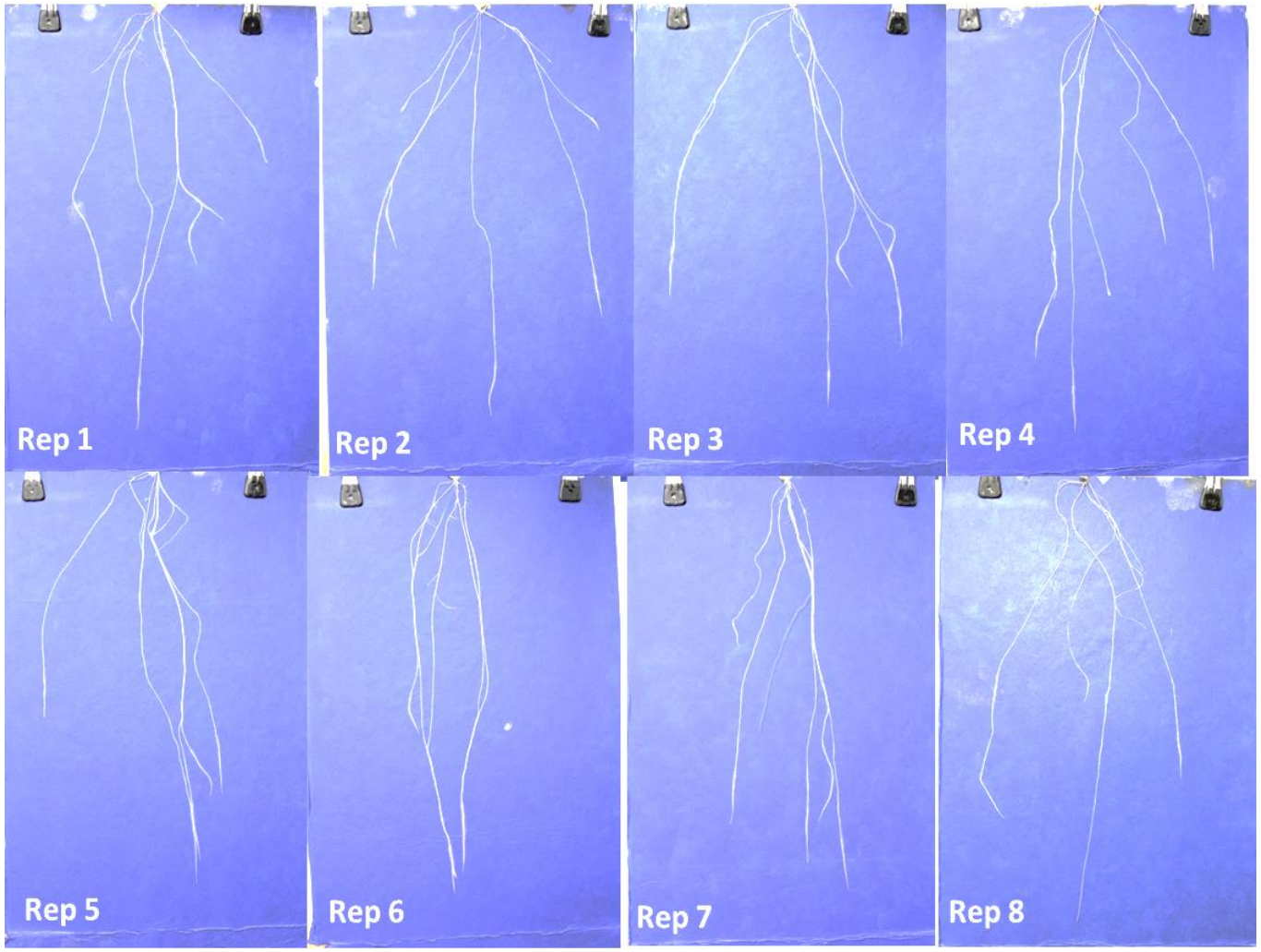
Appendix 19. Continued

D) OSU060



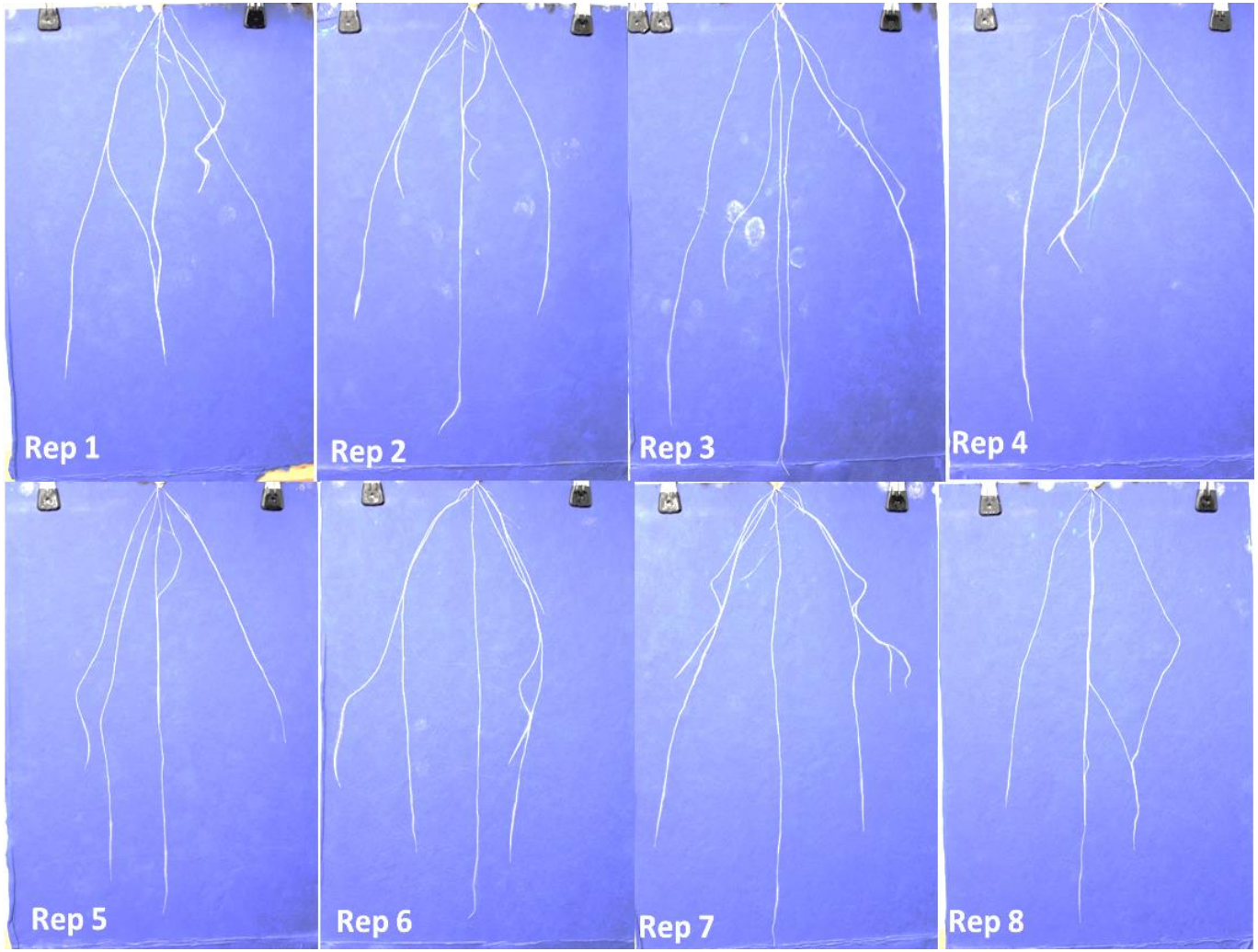
Appendix 19. Continued

E) OSU052



Appendix 19. Continued

F) OSU144



Appendix 20. Manuscript

Morphological and genetic characterisation of the root system architecture of selected barley Recombinant Chromosome Substitution Lines using an integrated phenotyping pipeline

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Abstract

Discoveries on the genetics of resource acquisition efficiency have now become limited by the ability to phenotype living plant roots with sufficient throughput. This paper presents a root phenotyping pipeline that combines a pouch system for time lapse root imaging and algorithms for model-based extraction of root growth parameters. The system was applied to a subset of barley Recombinant Chromosome Substitution Lines (RCSLs) and a combinatorial approach was designed for fast identification of the regions of the genome that contributes the most to variations in root system architecture (RSA). Results showed there is a strong genotypic variation in the root growth parameters within the set of genotypes studied. The chromosomal regions associated with primary root growth were different from the regions of the genome associated with changes in lateral root growth. The pipeline presented here could potentially accelerate the mapping of root QTL and assist breeding for novel crops with improved water and nutrient uptake efficiency.

Key words: root, phenotyping, QTL, barley, RCSL, growth parameters

Introduction

Profitability in modern agriculture relies heavily on the supply of water and fertiliser to maximise crop yield (Boserup, 2005). The current agro-economic model is now under increased scrutiny not only because of the damage it causes to the environment (Secchi *et al.*, 2007), but also because of its possible vulnerability to climate changes (Letter *et al.*, 2003) and the increasing cost and scarcity of some of the mineral compounds used in fertilisation (White *et al.*, 2012). Reducing the dependency of modern agriculture on water and fertilisers is a major undertaking, and it has been proposed that breeding programs should now focus on the development of crop varieties that are more efficient at capturing the soil resources (Lynch, 2011).

To date, the genetic improvement of crops for improved resource acquisition efficiency has proved challenging. A plant acquires water and mineral elements from the soil through a system of interconnected roots which is termed the Root System Architecture (RSA). The RSA is a complex object for breeders and geneticists to comprehend and utilise. The length and topological arrangement of roots within the RSA is dynamic because growth and lifetime of individual roots is controlled by a combination of developmental, physiological and environmental signals perceived by the plant (Forde & Lorenzo, 2001; Wilkinson & Davies, 2002; Bingham *et al.,* 2010). The development of RSAs is also very stochastic (Forde, 2009) and statistical characterisation of root traits and growth parameters usually requires large replication numbers (Adu *et al.,* 2014), observations in soil are destructive and labour intensive (do Rosario *et al.*, 2000), and *in situ* measurement techniques, partial (Nagel *et al.*, 2012). Some progress has been achieved in the understanding of genetic control of RSA and its potential for breeding. For example recently, a QTL controlling root growth angle in rice, Deeper Rooting 1 (*DRO1*), has been characterised and cloned (Uga *et al.*, 2013; Arai-Sanoh *et al.*, 2014). Nevertheless, major constraints for genetic studies in RSA persist. Because root traits are greatly affected by the environment, their heritabilities in many cases are low compared to shoot traits (Courtois *et al.*, 2009). Although genotypic variability is found for root traits in controlled conditions, few QTL have been identified and none have been used in breeding (de Dorlodot *et al.,* 2007). QTLs should generally be validated in field conditions before using a marker assisted selection (MAS, Comas *et al.*, 2013) but root traits measured *in situ* are not always directly related to field performance. Hence, root QTL studies face limitations that need to be overcome through improved technological and analytic approaches able to dissect the genetic control of relevant RSA parameters for the development of more efficient crops.

There is great hope that technological development in root phenotyping systems could unlock some of these challenges. Traditionally, root phenotyping is achieved in the field using either soil coring or shovelomics. Soil columns are extracted from the field, roots contained in the soil columns are washed, and usually image analysis software is used to measure total root length in the sample (Watt *et al.,* 2005). On the other hand shovelomics relies on measurement of the crown roots of the plant to describe parameters such as root gravitropism in the field (Trachsel *et al.*, 2011). These methods provide root data grown in their natural environment, but the measurements are destructive and time consuming. For these reasons, non-destructive methods have now become the preferred approach to study roots (Downie *et al.*, 2015). Rhizotron tubes can be placed in the soil to observe roots *in situ* (Rewald & Ephrath, 2012), but the observations are limited and provide little statistically robust data. Lab based rhizotron boxes allow part of the root system to be observed through glass windows (Nagel *et al.*, 2012). Root growth can be monitored for long periods of time, and image acquisition can be automated. Unfortunately, throughput in

such systems does not match those obtained in the screening of shoot traits and only a fraction of the root system can be observed. Systems based on X-ray computed tomography allow *in situ* imaging in undisturbed soil (Mooney *et al.*, 2012), but the technique is expensive, and the image data produced can be difficult to analyse. Although the throughput of such systems is increasing it is still low when compared to other techniques. There are also various artificial media systems for phenotyping (Clark *et al.*, 2011; Topp *et al.*, 2013), but the resulting data has not always been tested with field data.

Since current technologies do not allow RSA measurements to combine throughput with sufficient architectural resolution (de Dorlodot *et al.*, 2007), traditional approaches to QTL mapping are limited. One possible approach to overcome the throughput resolution tradeoff is to develop mapping populations for which identification of QTL require fewer genotypes, and to use such simplified root phenotyping systems where measurements are more easily acquired and automated. This paper illustrates this concept using a set of barley Recombinant Chromosome Substitution Lines (RCSLs, Matus *et al.*, 2003) with an accelerated root phenotyping pipeline. The study i) establishes a simple experimental system suitable to the screening of large numbers of barley plants at seedling stage; ii) uses a model-based approach to extract growth parameters for seminal and lateral roots from a time-lapse dataset; iii) assesses the effectiveness of the method for selecting lines contributing genetic variability for breeding programmes; and iv) explores new ways to identify regions of the chromosome that are linked to rooting traits in a subset of RCSL genotype.

Material and Methods

Plant material

Five barley genotypes were chosen from a set of Recombinant Chromosome Substitution Lines (RCSLs, Figure 1). The RCSLs were derived from an initial cross between a cultivated parent (cv. Harrington) and a naturally drought tolerant wild donor from the Fertile Crescent as described previously (Matus *et al.*, 2003). Selection of the sub-set of genotypes was based on a previous assessment of the impact of drought on yield across two growing seasons during field trials (De La Fuente Canto et al, unpublished). Contrasting lines were selected: OSU044 and OSU048 showed a weak performance but stable across water treatments (stable RCSLs), OSU144 and OSU052 produced large yield potential in favourable conditions but under drought their yield was significantly reduced (sensitive RCSLs) and finally, cv Harrington was chosen as control elite variety for the RCSLs and OSU060 as a line which performance was intermediate and similar to the performance of cv. Harrington.

Experimental system

Plants were grown in a controlled environment in a 2D pouch and wick system (Liao *et al.*, 2001; Hund *et al.*, 2009). Seeds with uniform size were surface sterilized by a vapour-phase sterilisation method using 100 ml sodium hypochlorite 4.5% and 5 ml concentrated HCl. The seeds were placed in opened Falcon tubes and treated for an hour with chlorine fumes inside a desiccator jar placed in a fume hood. Sterilised seeds were sown on 10x10 cm germination paper (Anchor Paper, St. Paul, MN, USA) moistened with sterile distilled water, placed in a Petri dishes and maintained vertically in a Qualicool^{TM} cooled incubator for two days at 20^{o}C with no light. Three days after sowing (DAS), seedlings of similar size were transferred to large sheets of germination paper (29.7 x 52 cm)pre-soaked with the nutrient solution, described below. Seedlings were held on the germination paper between an A3 size clear-Perspex plate and a 240 micron thick acetate sheet.

Each germinated seed was placed in a slit at the top of the germination paper and glued to the plate with a drop of diluted Solvite wallpaper paste (Henkel Limited, Winsford Cheshire, UK). Two foldback clips were attached to the sides of the plate and a clip hanger was used to hold the top of the plate. Each plate was wrapped in aluminium foil to protect the roots from light and suspended into plastic boxes (60cm x 68 cm x 46.5 cm) containing 30 L of nutrient solution into which only approximately 10 cm of the germination paper was submerged (Figure 1). The nutrient solution was constantly aerated with a pneumatic pump.

The same nutrient solution was used to soak the germination paper and to fill in the plastic containers. The nutrient solution was prepared with deionized water and contained 300mM NH_4Cl, 400mM $Ca(NO_3)_2$, 400mM KNO_3, 300mM $MgSO_4$, 100mM FeEDTA, 1M KH_2PO_4, 6mM $MnCl_2$, 23mM H_3BO_3, 0.6mM $ZnCl_2$, 1.6mM $CuSO_4$, 1mM Na_2MoO_4, 1mM $CoCl_2$. The pH was adjusted to 5.5 at the start of the experiment using NaOH and the nutrient solution was replaced every four days. Eight replicates of each genotype were distributed in four plastic boxes, two complete replicates per box. Plants were grown for 15 days in a growth room under a 16/8 h day/night cycle at a constant temperature of 15^{o}C and 60% relative humidity approximately. Average light intensity during the day hours was 80 μmol m^{-2} s^{-1} at plant height.

Phenotyping pipeline

1. Image acquisition

Pictures of each plate were taken every two days from day 2 to day 16 of the experiment with a Canon EOS 550D camera fixed on a tripod set on autofocus mode at a distance of 1 meter from the germination paper. The plate was hung in an easel with a 1 m working distance. The aluminium foil and acetate sheet were removed for taking pictures and, before putting them back, the germination paper was sprayed with approximately 1ml of the nutrient solution to ensure a homogeneous diffusion of the nutrients in the root system growing media.

2. Harvest

After the last image, 18 day-old seedlings were removed from the plates. Shoots were excised from the roots and fresh weight of the shoots was recorded. Roots were detached from the germination paper and stored at room temperature in 50% Ethanol until scanning. Roots were scanned (400dpi) using an Epson Expression 10000XL professional DIN A3 scanner (Seiko Epson Corporation, Japan). Root length and average root diameter were analysed using WinRHIZO (Regent Instruments, Quebec, Canada). Shoots and roots were dried at 60 degree C for 72 hours for determining the dry weight (DW).

3. Image processing

Image data were analysed through manual tracing of individual root trajectory using a liyama ProLite T2735MSC touch screen and Fiji software (Schindelin *et al.*, 2012). Raw images were first transformed into 8-bit grayscale images. For each genotype, the elongation rate of seminal and lateral roots as well as the branching rate of seminals were analysed on two time-steps, from day 2 to day 10 and from day 10 to day 16 of growth. Tracing was obtained using the freehand tool because many lateral roots were too small to be analysed efficiently using automated software such as Smartroot (Lobet *et al.*, 2011). ROI files produced for seminal and lateral roots of all the replicates for each genotype were then processed by a custom macro so that the pixel coordinates of all roots in the images were exported in text files. Direct estimation of growth parameters for each genotype over these two growth intervals used formula derived by Hackett and Rose (1972):

Equation 1

$$e^{(0)}(t) = \frac{l^{(0)}(t+dt) - l^{(0)}(t)}{n^{(0)}dt}$$

$$b_r^{(0)}(t) = \frac{n^{(1)}(t+dt) - n^{(1)}(t)}{dt - T}$$

$$e^{(1)}(t) = \frac{2l^{(1)}(t+dt) - l^{(1)}(t)}{n^{(0)}b^{(0)}(dt-T)^2}$$

here $e^{(0)}(t)$ and $e^{(1)}(t)$ (cm d^{-1}) are the elongation rate for respectively the seminal and lateral roots and $b_r^{(0)}(t)$ (d^{-1}) is the branching rate of lateral roots. The parameter *dt* indicates the duration of the examined growth interval of 8 days (day 2 to day 10) and 6 days (day 10 to day 16) respectively, while $l^{(0)}(t)$ (cm) and $l^{(1)}(t)$ (cm) is the total seminal and lateral root length at time *t.* The number of seminals is denoted by $n^{(0)}(t)$, the total number of laterals is denoted by $n^{(1)}(t)$. Since the number of seminal roots for the replicates of each genotype increased with time, $n^{(0)}$ was taken as the mean number of seminal roots during a given time interval where growth parameters were determined. For lateral roots, there was a time delay between the emergence of seminal and the emergence of lateral roots. The parameter *T (d)* is therefore the time it takes for lateral roots to emerge from the primary root. In this experiment, it applied only to the first timestep (day 2 - day 10), since after 8 days, laterals had emerged from all primary roots and was evaluated as the mean value of the time delay observed among the replicates of a single genotype.

Gravitropic rate was determined using stacked images from day 2 and day 4. In this case the images were first registered using the plugin Align Image by line ROI (Schindelin *et al.*, 2012). Registration of images from day 2 and day 4 used a line ROI with both ends of the ROI corresponding to the top and bottom of the slit. Two types of angles were recorded for these images. First the angle (α) of the root at day 2 was measured using the Straight Line ROI. Then, the change in angle ($d\alpha$) taking place for the same root between day 2 and day 4 was recorded using Segmented Line ROI and angle measurement. Three randomly selected seminal roots of each plate were measured. The gravitropic rate parameter ($g^{(0)}$) is defined as the fraction of the decrease in angle per unit time and it was determined for each genotype using the information gathered for a total of 24 seminal roots as follows:

Equation 2

$$g^{(0)}(t) = \frac{\alpha(t) - \alpha(t + dt)}{\alpha dt}$$

where *dt* is equal to 2, since the change in angle was measured for an interval of 2 days.

4. Statistical analysis

Statistical analysis of the genotypic effects on root traits measured was performed using a two factorial mixed model considering the genotype, the time-step (day 2 to day 10, day 10 to day 16 of the experiment) and their interaction as fixed effects. The experimental replicate was considered as the random effect. Genstat 17th Edition (VSN International, UK) was used for this analysis.

5. Combinatorial Quantitative Trait Loci Mapping (C-QTL)

In order to exploit the structure of chromosomal insertions contained in RCSL, a theoretical framework termed Combinatorial Quantitative Trait Loci (C-QTL) was developed. Within this new framework, the ith plant P_i is defined by its genetic makeup $G^i = \{g_1^i, g_2^i, \ldots g_n^i\}$ with i ≤ s, such that g_k^i takes the value 0 if the kth marker is that of the elite line and g_k^i takes the value of 1 if the kth marker is that of the exotic line. The ith genotype is also defined by its phenotype φi which is the quantitative traits corresponding to the genetic make up G^i. We therefore assume genotypes and phenotypes are related according to the following probabilistic model:

Equation 3

$$P(\varphi^i < x) = \int_{-\infty}^{x} N\left(x - a^i - \sum_{k \leq n} b_k g_k^i, \sigma\right) dx$$

where φ^i is considered to be normally distributed, a^i is the mean trait value observed on the modern variety and b_k is the effect of the ith marker on the genotype, and *s* is the standard deviation of the residual. *N* is the Gaussian distribution function. If two groups of distinct genotypes U_1 and U_2 are obtained, then variations between and within groups can be exploited to score each region of the genome using the following formula:

Equation 4

$$D_k^{1,2} = \delta_k^{1,2}\left(\frac{1}{n_1}\sum_{i\in U1}\varphi^i - \frac{1}{n_2}\sum_{j\in U2}\varphi^j\right)$$

n_1 and n_2 are the number of genotypes in U_1 and U_2 respectively and $\delta_k^{12} = 1\ if\ g_k^i \neq g_k^j$ for any $i \in G1$ and $j \in G2$ and $\delta_k^{12} = 0$ otherwise. It can be shown that D_k is an estimator of the genetic effect of loci k and it can be shown also that the bias is a function of the fraction of wild introgressions and cultivated introgressions from the other loci of the genome. For example, a population of genotypes with a full factorial combination of introgressions, the bias is 0 because there is always the same number of wild and modern introgressions in the population. It can also be shown that when wild introgressions do not overlap, the bias declines as a function of 1/n. It is interesting also to consider the lack of effect of certain loci on the plant traits. This is determined using the formula

Equation 5

$$E_k = \sqrt{max_{r=1,2}\left(\frac{\gamma_k^r}{n_r}\left(\sum_{i\in Ur}\varphi^i - \varphi^r\right)^2\right)}$$

where γ_k^r=1 if there exists two genotypes P_i and P_j in U_r such that $g_k^i \neq g_k^j$ and $\gamma_k^r = 0$ otherwise. E_k is therefore an estimate of the standard error of the mean within groups of genotypes.

Since there are many possible groupings on which to carry out such analysis, a logical and computationally efficient way to process the entire dataset is to use a clustering algorithm to group genotypes based on their similarity and to cumulate the indicators D_k and E_k on the possible set of clusters identified. The following formula is therefore obtained for scoring individual markers:

Equation 6

$$I_{C-QTL} = \left\{\frac{1}{n_{clusters}-1}\sum_{k=3}^{k\leq n_{clusters}}\left[\frac{1}{{n_{clusters}}^2 - n_{clusters}}\left(\sum_{i,j\leq n_{clusters}} D_k^{i,j}\right) - E_k\right], \quad k = 1,\ldots,n\right\}$$

C-QTL analyses were run on all four root growth parameters: the elongation rate of seminal root $e^{(0)}$, the elongation of lateral roots $e^{(1)}$, the branching rate $b_r^{(0)}$, and the gravitropic rate $g^{(0)}$. The data was transformed so that the value of each of these growth parameters had zero means and variance of 1. Clusters were created using the Agglomerative Clustering from the Scikit library (Pedregosa *et al.*, 2011).

6. Description of the change of the root system over time using a time-delay density based model

Growth equations were used to analyse growth patterns observed experimentally. The mathematical framework builds on the work proposed in Kalogiros *et al.* (2016) where root systems were modelled as a continuum and changes in the architecture of the root system over time were mathematically described with partial differential equations. The initial model was extended so that it could be used to extract growth parameters from time-lapse data. Modifications included time-varying growth parameters to characterise the changes in growth patterns over time allowing the pattern of the time delay in the emergence of lateral roots to be consistent with the time-lapse data considered.

Root density distributions are functions depending on the horizontal distance (x), depth (y) and root angle (α) which was defined with respect to the vertical axis. Therefore, at any point (x, y, α) the number of root tips per unit volume change according to the main conservation equation:

Equation 7

$$\frac{\partial \rho_{\mathrm{a}}^{(i)}}{\partial t} + \nabla \cdot e^{(i)} \rho_{\mathrm{a}}^{(i)} (\sin\alpha\,, \cos\alpha\,, -g^0\alpha) = b^{(i)}, \text{ with } i \geq 1$$

The index (i) describes the type of root so that seminal roots are denoted with the index 0 and lateral roots are denoted with the index 1. $\rho_{\mathrm{a}}^{(i)}$ (cm^{-2}) is the root tip density and $\frac{\partial \rho_{\mathrm{a}}^{(i)}}{\partial t}$ is the change with time of the root tip density. The operator $\nabla \cdot$ is the gradient of the root tip distribution function with respect to the independent variables x, y, α and $e^{(i)}(t)$ $(cm\, d^{-1})$ and $g^{(i)}(t)$ (d^{-1}) and $b^{(i)}(t)$ $(cm^{-2}d^{-1})$ describe respectively the elongation rate, gravitropic rate and the volumetric branching rate (termed also branching rate in the following sections) as functions of time. Since only seminal roots emerged from the base of the root system during the experiment, $b^{(0)} = 0$. For lateral roots, the branching rate is not zero and is specified as

Equation 8

$$b^{(i)}(x,y,a,t) = \frac{1}{2} b_r^{(i-1)} \left[\rho_{\mathrm{a}}^{(i-1)} \left(x, y, a + b_a^{(i)}, t - T^{(i)} \right) + \rho_{\mathrm{a}}^{(i-1)} \left(x, y, a - b_a^{(i)}, t - T^{(i)} \right) \right], \text{ with } i \geq 1,$$

where *T (d)* is the time delay observed before the emergence of the first lateral root, $b_r^{(i-1)}(d^{-1})$ is the seminal root branching rate and $b_a^{(i)}$ is the branching angle. In this setting, the root length density distribution $\rho_l^{(0)}$ and $\rho_l^{(1)}$ are derived from the root tip density distribution as $\int e^{(0)}(t)\, \rho_a^{(0)}$ and $\int e^{(1)}(t)\, \rho_a^{(1)}$, respectively. Solutions to equations 7 and 8 were obtained with an upwind finite volume solver with minmod flux limiters.

7. Spatial and temporal mapping of the root system architecture using density functions

In the next stage, the root tracing data was transformed into root length density so that model predictions could be compared directly to experimental data. The ROI's lists of pixels describing root trajectories were first processed to extract lists of root segments, their spatial coordinates, the length of the segment and its angle. Length density distribution functions were then determined using a kernel-based density estimation method. The method followed the principles of Kalogiros *et al.* (2016) but in this study, it was applied to pixel data directly and at different times during the experiment (day 2, day 10 and day 16). Kernel functions were fitted on data by the adjustment the band width *k* of the kernel function (here a Gaussian function). The heterogeneity of the distribution of root segments in space is a main challenge in order to achieve a good fit, because the data point distribution is dense along a root and sparse between roots. In this case, it is advantageous to consider groups of segments belonging to a single root (V-fold grouping) and apply cross validation to these groups of roots instead of separate random data points (Kalogiros *et al.*, 2016).

In a time-lapse dataset, both the number of root segments and the volume explored by roots increases with time. These two factors have opposite effect on the optimal *k*, with number of segments lowering *k* values and volume increasing *k* values. Overall, *k* values always increase because number of points increase linearly with time, but the volume increases as a power function of time. In order to simplify the analysis, we choose the largest optimal value of *k* which was always on the last day of growth. Hence, the bandwidth *k* was first evaluated on the last day of the experiment (day 16) and the same value was used for estimating the root length density for the other time points of the experiment. Finally, the seminal root length density distribution maps on each day were aligned with respect to the midpoint of the horizontal distance of the plane. (Figure 1; Step C).

8. Estimation of time-dependent model parameters from time-lapse data

Fitting the root length density model to time-lapse data is achieved through a series of stepwise optimisation sub-problems that were solved in a well-defined sequence. First, the tracings on day 2 were used to determine initial root tip density and root length density. The length density was initiated directly using the kernel-based density estimation. Since it is not possible to distinguish root tips from and root bases from the tracings, the length density at day 2 was also used to determine the root tip density.

Equation 9

$$\rho_a^{(0)}(x,y,a,2) = n^{(0)} \frac{\hat{\rho}_l^{(0)}(x,y,a,2)}{\int \hat{\rho}_l^{(0)}(x,y,a,2)dxdyd\alpha}$$

with $\hat{\rho}_l^{(0)}$ denotes the root length density distribution function estimated using kernel-based methods from the experimental data made available on day 2. The same data was used to determine the initial value of the root length density at the beginning of the simulation. The optimal set of growth parameters were obtained using the following robust error function:

Equation 10

$$E^{(i)} = \int_V \hat{\rho}_l^{(i)^2} \left(\rho_l^{(i)} - \hat{\rho}_l^{(i)}\right)^2 dxdyda + \left(\int_V \left(\rho_l^{(i)} - \hat{\rho}_l^{(i)}\right) dxdyda\right)^2$$

The first integral term accounts for local differences between the observed $\hat{\rho}_l^{(i)}$ and predicted $\rho_l^{(i)}$root length density. It is a modification of the mean square error that reduces the dependency of the error on areas of relatively low root length density in the spatial domain. The second term of the error accounts for the differences in total root length density. The Nelder-Mead optimisation algorithm was used to obtain parameter values $e^{(0)}, g^{(0)}$. Lateral root growth parameter $b_r^{(0)}, e^{(1)}$ were obtained in a second stage.

Model fitting in each of the time steps following initiation of the model was treated as a distinct optimisation sub-problem. For each of the subsequent time-steps, Both the model parameters and the root length and root tip density were initiated from the final state of the simulations of the previous optimisation step. The time increment for the numerical solver was set constant for the total duration of the experiment. In order to insure the Courant–Friedrichs–Lewy condition for the stability of the finite volume solutions to be obtained, the constant time step was used.

9. Fitting model parameters on data

The parameter extraction pipeline was benchmarked on simulated data for which growth parameters were known. The model used to establish the benchmark consisted of equations 7 and 8 without lateral roots, for which the elongation rate $e^{(0)}$ was either a linearly decreasing function of time or exponentially decreasing function of time. The data generated by these models were used in the optimisation algorithm described above, and the results were compared with the model parameters used to generate the target root length density function. In a second step, the optimisation pipeline was applied to the entire root tracing dataset. For each time interval the Model Elasticity Value (MEV) of the error was determined as the percentage increase in the error induced by a 1% increase in each model parameter. Confidence intervals for model parameters were estimated using the V-fold bootstrap method proposed in (Kalogiros *et al*, 2016),

Results and discussion

Integrated phenotyping and computational methods allow automated extraction of growth parameters

To date, the identification of root QTL has usually been limited by the throughput of root phenotyping systems, the lack of specific mapping populations with known genotypic variation in root traits, and the need for a suitable theoretical framework to describe the changes within the root system during growth. This study proposes to overcome these limitations through a dedicated phenotyping and data processing pipeline combining 1) a simple pouch and wick phenotyping system that is scalable and amenable to acquisition of data, 2) a selection of RCSLs where identification of loci can be achieved with fewer genotypes, 3) a model-based framework for the estimation of growth parameters and 4) a new marker scoring system termed Combinatorial QTL (C-QTL).

The approach based on a pouch and wick system was tailored for the observation of barley roots in RCSL seedlings of up to 18 days-old and image acquisition using a DSLR camera. After sixteen days of growth, seminal roots fitted tightly within the boundaries of the A3 sized pouches, without touching any of the edges. Such root phenotyping systems on germination paper have been successfully used in cereal crop plants such as maize (Hund *et al.*, 2009) and wheat (Atkinson *et al.*, 2015) and many other crop species (Adu *et al.*, 2014). The results presented here confirm the system is suitable to study the root system architecture of barley seedlings. Elongation rate of seminal roots (approximately 1 - 2.5 cm d^{-1}) were

similar to those measured in soil (Dupuy *et al.*, 2010; Valentine *et al.*, 2012), in hydroponics (Rose, 1983) or in gels (Shelden *et al.*, 2013). Visual inspections of the plant showed vigorous growth and no signs of stress and mineral deficiencies. Other simple phenotyping systems have been used in the past e.g. gel chambers (Bengough *et al.*, 2004), or imaging at the surface of transparent cylinders (Kristensen & Thorup-Kristensen, 2004) or gel systems (Topp *et al.*, 2013), but cost and the time for sample preparation in such systems is higher.

The assistance provided by models and computational tools allowed accelerated acquisition of root growth parameters. Image registration was the first step of the analysis because the position of the samples in the image varied due to the approximate placement of samples in front of the camera. Once a time lapse sequence of images is aligned with respect to a reference image, the trajectory of individual roots can be recorded in a numeric data format. Many software are available to carry out this task (Armengaud *et al.*, 2009; Lobet *et al.*, 2011; Pound *et al.*, 2013). Most of them rely on the manual placement of markers on the image. Markers are then used as input to an algorithm that progresses along the root until stopping criteria are met. The data generated by this study however contained many small lateral roots for which the placement of markers was difficult and slow. Semi-automated tracing provided limited advantage in speed due to the short length of the lateral roots. Preliminary tests carried out on the data showed no time was gained by using the SmartRoot software (Lobet *et al.*, 2011), and therefore manual tracing was used to analyse the images. Root length density maps were subsequently estimated using kernel based density estimators and V-fold cross validation. Unlike previous studies (Kalogiros *et al.*, 2016), the method was applied directly to pixel data derived from root tracings. Finally, the root growth model that best matched the root length density maps was determined using optimisation algorithms and the elongation rates, branching rates and gravitropic rates were obtained from the resulting model growth parameters for each genotype.

The pipeline was used successfully to extract the growth parameters from a selection of RCSL genotypes with contrasting response to drought. Results showed that the pipeline is amenable to automation because image data from the pouch and wick system was suitable to image analysis using SmartRoot, and there is great potential from next generation software to achieve such tracings with limited or manual interventions, e.g. combining root tip detection (Kumar *et al.*, 2014) with optimal path search (Pound *et al.*, 2013), active contour (Makowski *et al.*, 2002), or tracking algorithms (Mairhofer *et al.*, 2012). Subsequent processes for the extraction of growth parameters were fully automated. There is also potential for the phenotyping platform presented here to be made widely accessible to the wider community. All the materials used to assemble the pipeline were low cost, generic and easily acquired.

Software used to process the data was open source, including image analysis, modelling and optimisation software and this can be downloaded at www.archiroot.org.uk. Although the study focused on few selected genotypes, results showed the phenotypic pipeline is suitable to detect genotypic variations in rooting traits, and similar analyses could be carried out on larger number of genotypes simply by allowing for more pouches to be grown simultaneously during an experiment. This has been achieved in a recent study on Brassica genotypes (Thomas *et al.*, 2016).

Models allow accurate estimation of time varying growth parameters

Automation of the extraction of root growth parameters was achieved using models controlled by an optimisation algorithm. The optimisation algorithm was used to search for root growth parameter values that best mimic the experimental root length density profiles at different times during the experiment. To date, optimisation techniques have been used for model calibration (Reddy & Pachepsky, 2001), to predict for example the spread of roots through soil under different fertilisation regimes (Heinen *et al.*, 2003). The problem of extracting biologically meaningful information from data is more challenging because models can make accurate predictions without biologically meaningful parameters. Recent attempts to solve this problem have shown root growth rates can be estimated accurately when the root system is simple (Kalogiros *et al.*, 2016), but when more complex models are used the optimisation process is more challenging (Garré *et al.*, 2012) .

Here, the method proposed by (Kalogiros *et al.*, 2016) was extended to allow estimation of time varying growth parameters. The difficulty of such an extension is that parameters of the numerical algorithm for model simulation such as grid size, time increment, or the size of the data buffer for simulation of delays, are dependent on both the duration of growth and the observed root system through the bandwidth k of the kernel estimator. In order to run the optimisation algorithm on time lapse data, the simulation time was cut into large blocks of time increments that matched the days of imaging (Fig 2A). At the end of each block of time, the optimisation algorithm was reset and the model was initiated with the root tip density from the previous block of time. In order to maintain a constant grid size, the bandwidth of the density estimation was determined on the last time-step of the experiment (largest k value for each genotype). The new method was tested using simulated data as a template for the optimisation algorithm, and results showed the method was able to retrieve linear and non-linear time varying growth parameters from the template data (Fig 2B).

The growth parameters obtained on the experimental data were also compared to direct measurements (Fig 3). Model outputs were visually compared to experimental output (Fig 4) to ensure a good match was obtained between predicted and measured root length density distribution. Strong correlations were observed between direct measurements of growth parameters and model based estimations of growth parameters (Fig 3). Correlations were greater for the elongation of seminal roots (Fig 3A) than for the elongation and the branching rate of laterals (Fig 3C and 3D). However, weaker correlations were observed for the elongation and branching rate of lateral roots due to the stochasticity of these parameters and this result was not related to the optimisation process (Table 1 and 2). Likewise, the gravitropic rate was more difficult to determine experimentally due to the stochasticity of the direction of growth. Direct estimation of gravitropic rate was obtained using the angle of roots at day 2 and day 4. However, correlation between the initial angle of the root and the magnitude of the change in angle were determined (Fig 3B). This allowed, for the first time, confirmation of linearity of the gravitropic response as was proposed in earlier theoretical studies (Dupuy *et al.*, 2010). However, this measure of the gravitropic rate may be of limited value because it was obtained at a fixed point in time. The measure is therefore more sensitive to root stochasticity and it may not be representative of the overall plant behaviour since the gravitropic rate may change with time. Results suggest that the global estimation of the gravitropic rate using the optimisation pipeline was more realistic (Table 1). Direct estimation of the gravitropic rate predicted genotype OSU060 to be more gravitropic than cv. Harrington whereas they were genetically and visually very similar (Fig 5). There was no major difference in the estimates of the gravitropic rate obtained from the optimisation pipeline for the duration of each growth period among genotypes.

Both genotypic and temporal factors affect root growth parameters

Recombinant Chromosome Substitution Lines with contrasting response to drought in field trials showed remarkable genotypic variations in the morphology of their root system at early growth stages. Seminal root elongation rate was the most discriminating variable across the RCSLs ($p<0.001$, Table 3). For instance, OSU048 (stable but limited yield performance) had a remarkably low and uniform elongation rate throughout the experiment (0.94 ± 0.04 cm d^{-1} and 1.13 ± 0.14 cm d^{-1} from day 2 to 10 and day 10 to 16 respectively, Fig 5). In contrast, OSU144 (sensitive but large yield potential) showed an overall decrease in elongation rate for the seminal roots, with a higher elongation rate from day 2 to 10 (2.8 ± 0.2 cm d^{-1}) than from day 10 to 16 (2.3 ± 0.1 cm d^{-1}). This trend was observed for all the genotypes except OSU048 and OSU044. Branching rate and lateral root elongation rate showed large variation at the genotype level due to the stochasticity of these growth parameters. For all the genotypes, the number of lateral roots emerged from day 2 to day 10 of the experiment was larger than the number of lateral roots that emerged from day 10 to 16. Genotypic differences were found for elongation rate of lateral roots ($p<0.05$, Table 3).

Lateral roots in OSU048 grew vigorously from day 10 to 16 (0.7±0.3 cm d^{-1}) and this resulted in a much larger total lateral root length at the end of the experiment (40.2±7.0 cm), compared to genotypes such as OSU044 (4.5±1.4 cm) and OSU052 (13.3±3.5 cm) which had a lateral root growth rate that was significantly lower (Fig 5). These results indicate that the genotypic response of the RCSL population to water deficit observed in the field (de La Fuente Canto 2016, unpublished) may be linked to genotypic variations in the root system architecture, and this variability can be observed at a seedling stage. OSU048 and OSU144 were the two most contrasting phenotypes with a final total root length of 159.4±10.7 cm and 284.5±23.8 cm respectively. OSU060 was selected because of the similarity of its performance to cv. Harrington in field conditions (de La Fuente Canto, in preparation), and results showed its growth parameters were comparable to cv. Harrington (Fig 5). This suggests the exotic introgressions present in OSU060 also had a negligible effect on the root system at this stage of development.

Comparative studies of modern and ancient crop varieties have suggested breeding has induced changes in the size and architecture of the root systems (Chloupek *et al.*, 2006). For example, modern barley cultivars were found to have a larger numbers of seminal roots with a wider angular spread of roots compared to their wild relatives (Bengough *et al.*, 2004). Since modern cultivars rely on a large supply of nutrients and water, it is possible, that their performance under reduced fertilisation and or irrigation is not often optimal (Letter *et al.*, 2003). To engineer crops systems that are efficient in low input cultivation conditions, it is probable that the roots of such new crops will need to acquire soil resources from different regions of the soil. For example, improving the rooting depth could be used for resistance to drought (Kato *et al.*, 2006), and enhanced lateral root development in the topsoil could provide better nutrient uptake efficiency (Lynch & Brown, 2002).

In this study, the variations observed in root growth parameters for the RCSL genotypes indicate the genetic background of wild barley could be used to modify the root system architecture of modern barley cultivars. For example, there was significant variation in root gravitropism and primary root elongation rate between the RCSL genotypes, and this could be exploited to create deep rooting genotypes. Del Pozo *et al.* (2012) found evidence suggesting segregation in the deep root phenotype within the RCSL population used for this study. The authors carried out a field trial and found that drought tolerant RCSLs had greater values of grain $\Delta^{13}C$ compared to cv. Harrington, which may indicate greater access to soil water during grain filling and a more extensive root system (Tambussi *et al.*, 2007). The differences found for root elongation rate and gravitropism at early stage of development in the RCSLs tested in the present study support this hypothesis since these two traits have been associated with deep rooting phenotype in cereal crops (Araki *et al.*, 2002). and it has been shown to be an important quantitative trait

to improve water uptake and yield under water stress in rice (Uga *et al.*, 2013) and maize (Hund *et al.*, 2009). There were also significant variations in the elongation rate and branching rate of lateral roots. Lateral roots are essential to the acquisition of nutrients because they allow intensive exploration of the subsoil and their ability to solubilize minerals adsorbed on the surface of soil particles. Lateral roots for example, have been shown to increase the uptake of immobile nutrients such as phosphorus (Lambers *et al.*, 2006).

Use of RCSLs for identification of root QTL

Because of the limited number of lines selected for this study, it was not possible to carry out a traditional QTL mapping analysis such as the composite interval mapping (CIM) used by Uga *et al.* (2013) in 117 rice RILs where *DRO1* was detected, or the multiple interval mapping (MIM) used by Chen *et al.* (2010) in a 134 F_4 barley mapping population where several root QTLs were identified. Instead, a combinatorial approach (C-QTL) was proposed to visualise broad regions of the genome associated with rooting traits. The method relies on the grouping of lines based on the similarity of their phenotypes. A metric was then computed for each marker, using variations observed between and within groups. Since there are many different ways of grouping genotypes, a cluster algorithm was used to create the sets of relevant groups on which the metric was cumulated. Since the metric accounts for both within group variability and between group variability, it can be used to outline regions of the genome that may be linked to variations in a quantitative trait, regions of the genome that varies without having an effect on a quantitative trait, and regions of the genome on which no information can be derived.

The C-QTL method described here has similarities with resampling techniques used in non-parametric statistics. For example, Bootstrapping uses random resampling of the data with replacement to produce simulated data of how an estimate varies, and to compute confidence intervals of estimates directly from these simulations (Efron and Tibshirani, 1994). Cross validation techniques employ a range of resampling schemes (leave one out, V-fold, Monte Carlo) for example to determine the log likelihood of a model (Burman, 1989). In a permutation test, samples are randomly rearranged between groups to assess the likelihood of the null hypothesis (Kim et al., 2000). The method also shares some similarities with single marker mapping (Geldermann et al., 1985) since the metric determined on two sets of genotypes is a direct estimate of the effect of the group of markers that makes the two groups genetically different. However, the C-QTL approach is different from these methods, in that the whole dataset is used in the simulations and it is the grouping of the data that is resampled to compute the net effect of a marker. Intuitively, the method provides an optimal way of grouping genotypes that minimize the number of computations while maximize the information contained in the metric. The method was extensively tested

on a larger selection of RCSL lines (De La Fuente Canto, in preparation) using heading date as a reference trait. The test showed C-QTL co-locate with key genomic regions associated with barley phenology. To date however, it is unclear how the resampling of the groups affects the bias and variance of the estimators of the marker effect, and how different ways of grouping genotypes could improve the quality of the estimates. Additional theoretical work is now required to further characterise the mathematical properties of C-QTL estimates. Further development could also expand the technique to include common statistics on the significance of the effects of markers. For example, permutation tests could be implemented in the C-QTL analyses to determine the statistical significance of QTLs identified (Doerge & Churchill, 1996), because they do not require *a priori* knowledge of the statistical distribution of the sample data.

C-QTL analyses provided a coarse but extensive map of the influence of wild barley chromosomal introgression on rooting traits (Fig. 6-7). Because of the small number of genotypes studied, only a few substitution segments from the wild genome were tested and associations for several root growth parameters are likely to co-vary with other unrelated markers (Fig. 1D). Regions associated with primary and lateral root elongation rates (Fig. 6 A, C) were mostly identical across the genome, with the highest scores recorded simultaneously on chromosomes 1H, 2H, 3H and 4H, moderate score values on chromosome 6H and no associations on chromosome 5H. In addition, small groups of markers on chromosomes 2H, 3H, and 7H appear to be solely associated with the elongation rate of seminal roots whereas a common group of markers on chromosome 4H was found to overlap with seminal elongation rate, gravitropism and branching rate. In particular, the wild barley introgression on chromosome 2H (68.6cM to 80.9cM) found on OSU048 could be linked in elongation rate. No QTLs have been reported in the literature for roots growth rate parameters in barley, but Chen et al (2010) and Arifuzzaman et al (2014) detected genomic regions on chromosomes 2H, 3H and 5H influencing root length. Both authors used populations derived from Israeli wild barley accessions in their studies and showed the potential of the unadapted genome to contribute favourable alleles to increase root length and so adaptation to water-limited environments.

Regions associated with gravitropic rate (Fig. 6B) and branching (Fig. 6D) rate were less significant than the associations found for elongation of primary and lateral roots. Two regions on chromosome 2H and 6H were uniquely associated with gravitropic rate, also a large group of markers chromosome 5H were found associated with the trait but with very low score. Recently, Robinson et al (2016) reported a major QTL associated with root spread on chromosome 5H using a double haploid population (ND24260 X Flagship). The authors found this region collocated with other QTL controlling seminal root number

which also mapped in the vicinity of aboveground quantitative traits related to drought adaptation in barley. A chromosomal region on 7H was associated solely with the elongation rate of lateral roots and a chromosomal region on 4H was associated only with the branching rate. No QTLs have been reported for this trait in previous studies. It is interesting to note also that the score of markers associated with the primary elongation rate and the branching rate varied strongly as a function of time (Fig. 7), whereas the score associated with the elongation rate of lateral root was more consistent at the different time steps.

Accurate identification of root loci from a small subset of RCSL genotypes is challenging. First results showed correlations exist between groups of markers because of limited number of genotypic combinations within the genome (Fig. 8). Physiological interactions are also likely to create natural correlations between several traits. It is often observed that elongation of primary and lateral roots are linked, for example, enhanced elongation of lateral roots coincides with a reduction in the growth of primary (Williamson et al., 2001). In order to overcome such limitations, it is important therefore, to optimize the distribution of wild introgressions within a selection of RCSL genotypes to be used in a study. An essential property to consider for the C-QTL approach is the balance between wild and cultivated introgressions within the selection of genotypes. An ideal set of lines would have introgressions arranged with minimum overlapping of segments and each marker would appear in exactly the same number of times in the set of genotypes. This is difficult to achieve practically because of the large number of genotypes that would be required. For example, with 10 segments a full factorial set of introgressions would require 210 to 1024 genotypes. A more straight forward and effective approach would be to phenotype introgression lines harbouring a unique exotic insert from the donor parent genome. Lines from the initial cross between cv. Scarlett X ISR42-8 (von Korff, Wang, Léon, & Pillen, 2004) have been further backcrossed to the recurrent parent and new subset of lines with unique introgressions have been used in root QTL mapping studies (Hoffmann et al. 2010; Naz et al. 2014). In this case, QTLs are located to the target segment making the introgression line significantly different from the donor parent and the results can be validated using a small number of introgression lines (Naz, Ehl, Pillen, & Léon, 2012). However, this approach is not suitable for groups of introgresion lines in earlier generations (BC2) since they contain several alien inserts in their genome. The C-QTL approach could aid the selection of target regions putatively associated with the trait for further experiments, optimising the number of introgression lines used and the backcross strategy to obtain near isogenic lines and ultimately identify the genes underlying the QTL.

Conclusion

Since root phenotyping is slow, successful mapping of QTLs is challenging. New approaches must be developed to overcome limitations due to the complexity of root systems. Here, we describe an approach that allows efficient measurement of root traits and potential identification of QTLs linked to these root traits. The study makes use of a selection of barley RCSLs with well-defined chromosomal

introgressions. The lines were grown in a simple root phenotyping system and a computational pipeline was developed to extract root growth parameters in a time lapse dataset. The next step is to design the next generation of RCSL lines and refine the chromosomal regions associated with root growth parameters.

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Figures

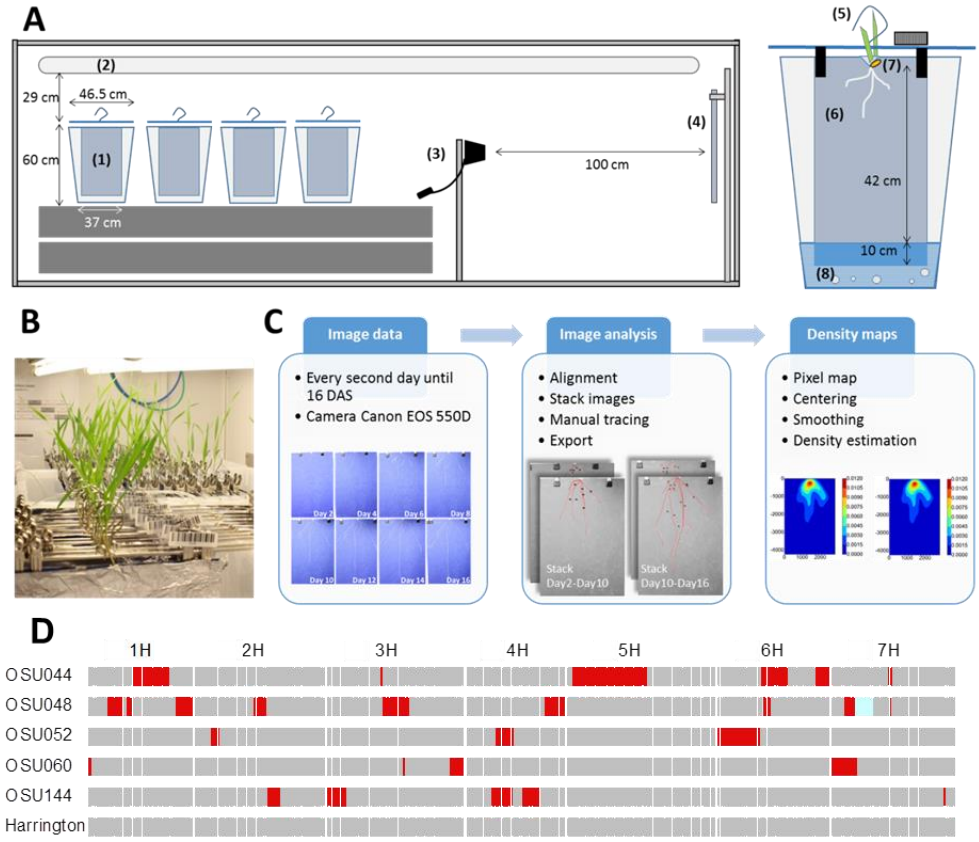

Figure 1. The root phenotyping study. A) Diagram of the pouch-and-wick experimental setup used to grow barley seedlings under controlled conditions. (1) Each bucket contained two experimental replicates (12 seedlings, one per plate). (2) Lighting consisted of fluorescent tube light placed at 29 cm above the buckets and (3) a Canon EOS 550D camera was used for image acquisition. The camera was placed on a tripod with a remote switch attached. (4) An easel was used to hold the samples at a reference position and (5) the clip hangers used to hold the samples on the easel were fitted with a barcode . (6) roots grew on A3 size clear-Perspex plate and acetate sheet with germination paper in between. Each plate was wrapped in foil and (7) seedlings were attached on a slit on top of the germination paper. (8) The nutrient solution was aerated with a pneumatic pump and 10 cm of the germination paper was submerged in nutrient solution. B) Picture of the experiment setup in the growth room. C) Diagram of the data processing pipeline. The raw phenotyping data consisted of images taken every two days for 16 days after sowing. The images were analysed using a series of steps including registration for aligning data with a reference image, stacking, tracing and exporting the pixel ROI data to files. Pixel ROI were then used to generate root density distribution maps for primary and lateral roots. This was done using kernel-based density distribution methods combined with a centering of the data with respect to the midpoint of the horizontal plane (position of the slit on the germination paper). D) Graphical representation of the genotypes of the 5 RCSLs used in the study and cv. Harrington. Dark red areas indicate the introgressions from the wild parent and light grey areas indicate the modern background. Missing marker data are indicated in light blue. Each chromosome is oriented with the left arms from the left.

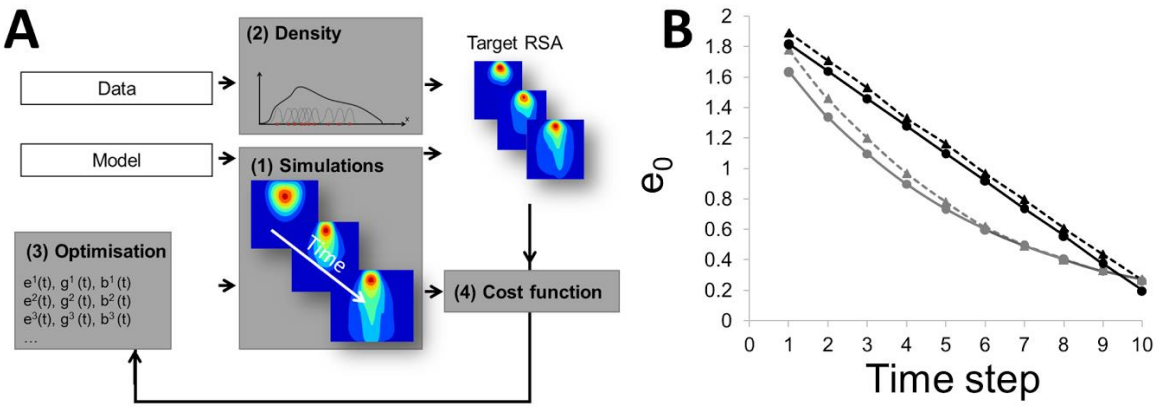

Figure 2. A) Diagram of the optimisation process for automatic identification of growth parameters over time. The target root system architecture (RSA) at specific time points results from the available experimentally observed time-lapse data or artificial data (model-generated data with model parameters known). A target root length density distribution function is derived from simulations with user-defined time-varying parameters for each time step (1) so that it is feasible for the estimated model parameters to be directly compared to target parameters over time. When dealing with experimental data, root density estimation methods (2) are applied to obtain the target RSA. Then, the optimal time-dependent model parameters are determined by applying a minimisation algorithm (3) that propose, at each time step, a set of new candidate model parameters. The new set of parameters is in a simulation and the results of this simulation is compared with the target root system using a cost function (4). The optimization procedures (3 and 4) are iterated until a convergence criterion is met. The output seminal root distributions with the estimated optimal parameters at a specific time step are used as the initial condition for the evaluation of the optimal model parameters at the next time step. B) The quality of the fit obtained with the optimisation algorithm was tested on simulated data using time-varying elongation rate e_0 of seminal roots (dashed lines with triangle markers). The optimisation algorithm could retrieve accurately the variations with time of the elongation rate (plain line with square markers) in both linear (black) and exponential (grey) decline.

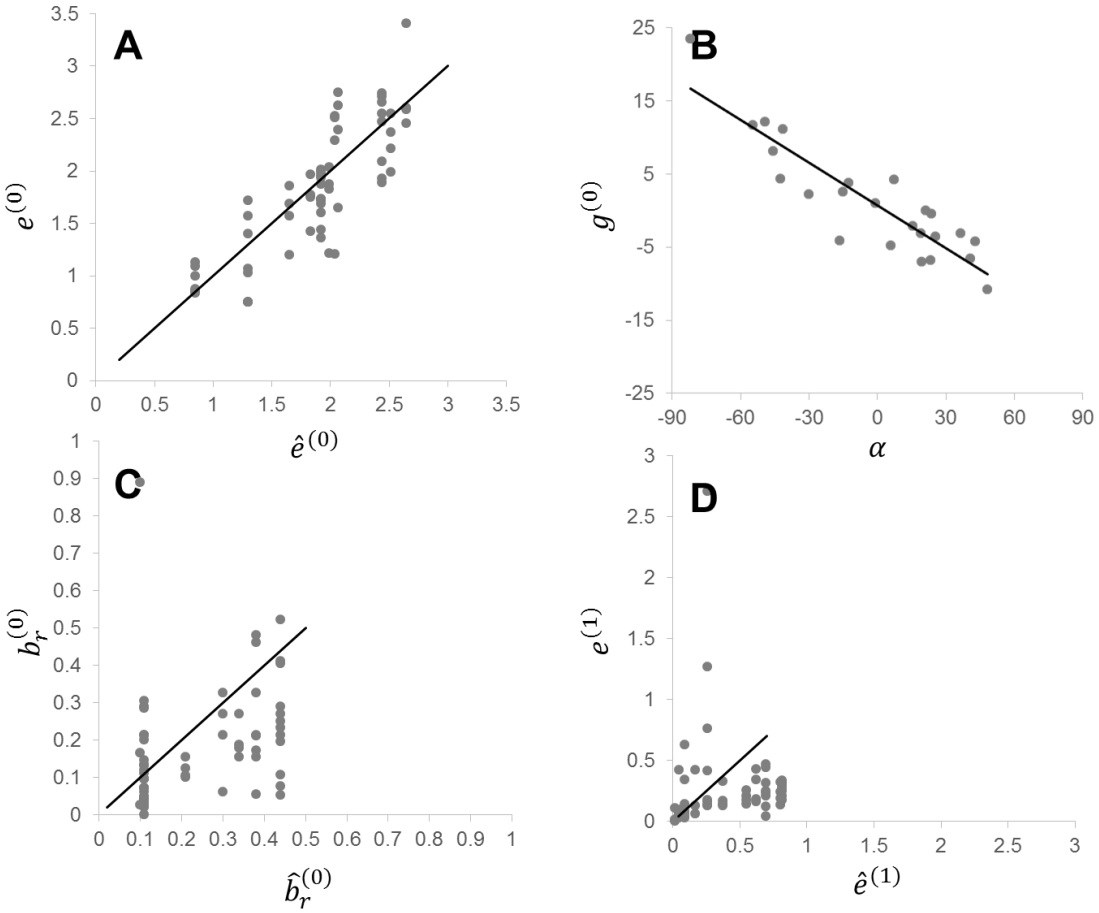

Figure 3. Comparison between automated extraction of model parameters and direct estimation from tracing data. Values on the x axis indicate the mean values for model based estimation of the growth parameter. Values on the y axis indicate the growth parameter obtained directly from manual tracing of the data. Data is presented for A) the elongation rate $e^{(0)}$ of seminal roots; B) the gravitropic rate $g^{(0)}$of seminal roots for the genotype OSU 048; C) the branching rate $b_r^{(0)}$of seminal roots; and D) the elongation rate $e^{(1)}$of lateral roots.

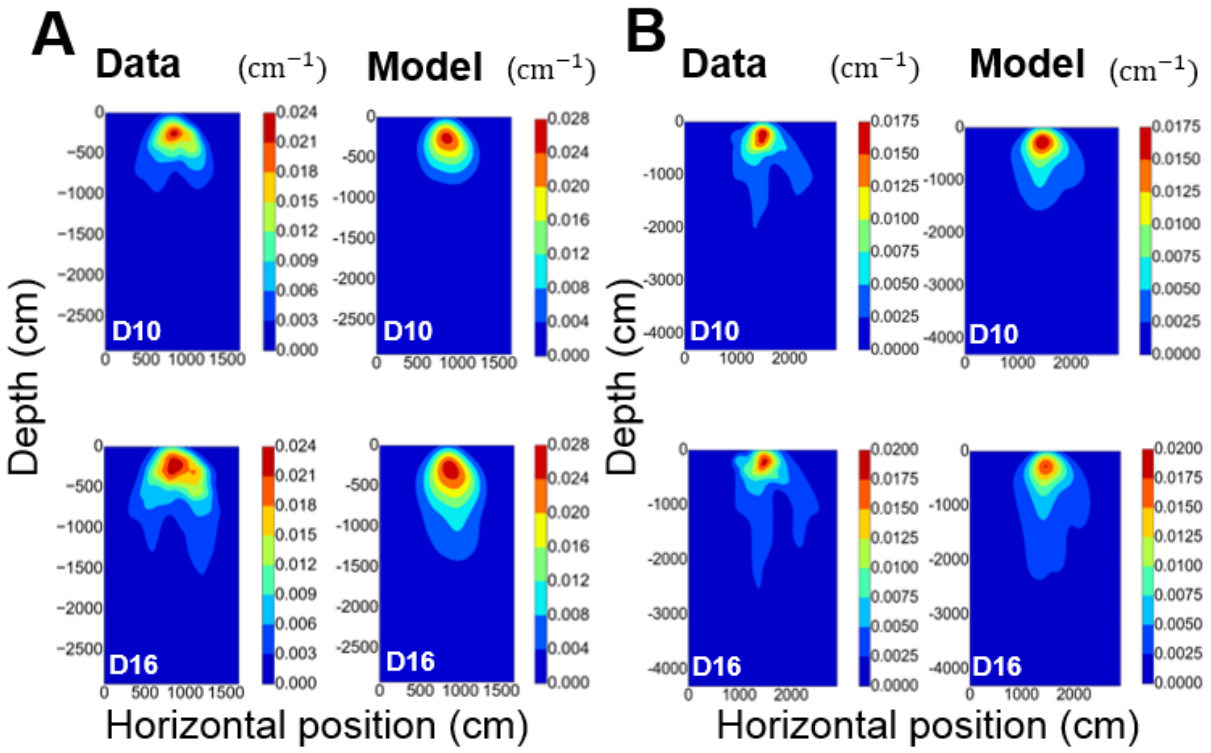

Figure 4. Comparison between predicted root length density distribution with optimal growth parameters and experimental root length density estimation determined using kernel density estimation method at Day 10 (D10) and Day 16 (D16) of the experiment. The data is presented for A) genotypes OSU 048; and B) cv. Harrington.

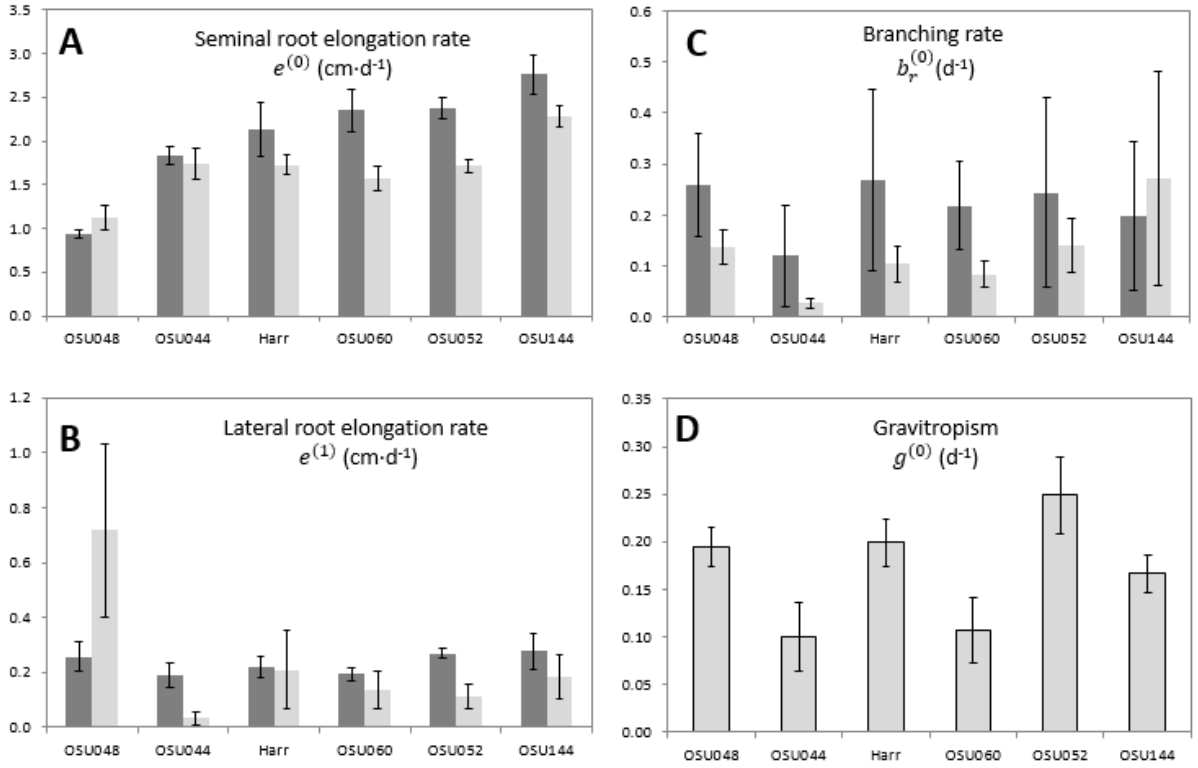

Figure 5. Variations in root growth parameters with time and as a function of genotype. Bar charts represent mean values (+/- SE) for A) seminal root elongation rate (cm d^{-1}); B) lateral root elongation rate (cm d^{-1}); C) branching rate (number of branches (root)/day). Growth parameters from Day 2 to Day 10 are plotted with dark grey shading, and growth parameters from Day 10 to Day 16 is plotted with light grey shading. D) Genotypes mean value for gravitropic rate measured from Day 2 to Day 4. Error bars represent standard error of the mean.

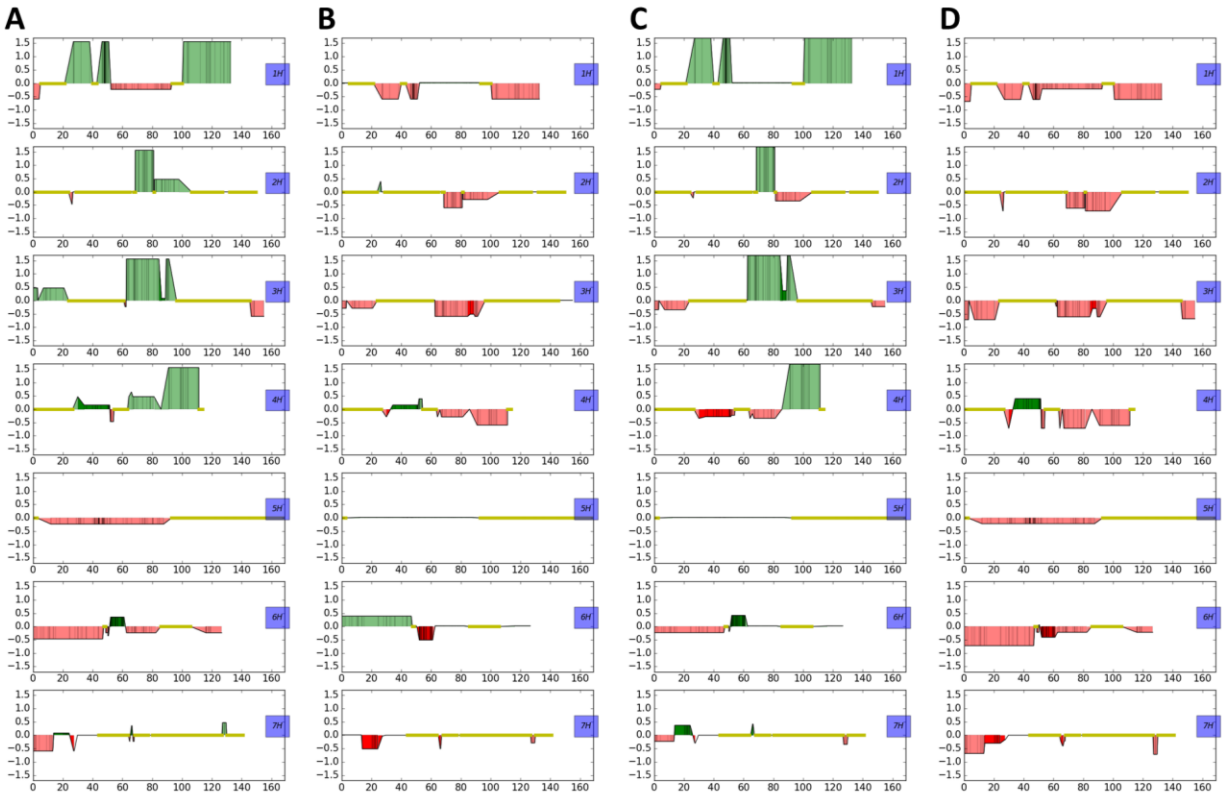

Figure 6. Chromosome regions associated with root elongation rates. Green areas of the graph indicate region of the genome for which variations are associated with changes in the quantitative trait. Red areas of the graph indicate regions of the genome which variations are not associated with variations in root traits. Darker regions (respectively green or red) indicate region where there is more chromosomal introgression for which estimates are likely to be more accurate. Horizontal lines in yellow indicate region of the genome for which no genetic variations are observed within the selection of genotypes studied. Chromosome regions associated with primary elongation rate A) and gravitropic rate B) lateral root elongation rate C) and branching rate D).

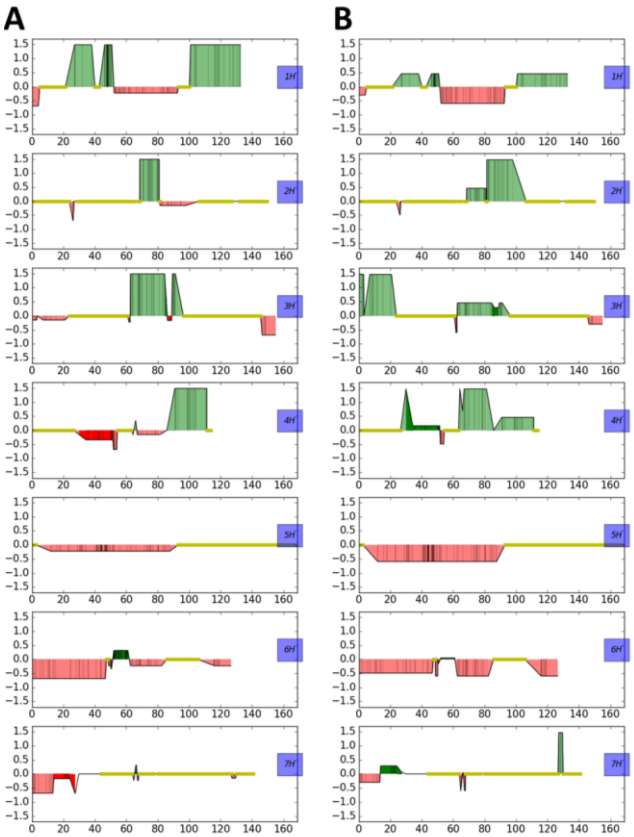

Figure 7. Change in regions associated with primary elongation rate with time. Chromosome regions associated with primary elongation rate at day 10 A) and primary elongation rate at day 16 B) showed a few differences in chromosomes 2H, 3H, 4H and 7H.

Tables

Table 1. Estimated root growth parameters for primary roots using the optimisation pipeline (Figure 2) and comparison between measured and predicted total root length.

Genotype	Elongation $(cm \cdot s^{-1})$		Gravitropism (s^{-1})		Total root length(cm)		Predicted total root length(cm)	
	Δt_1	Δt_2	Δt_1	Δt_2	Δt_1	Δt_2	Δt_1	Δt_2
OSU_048	0.85	1.30	0.258	0.224	68.30	107.42	68.91	108.72
OSU_044	1.92	1.99	0.168	0.162	109.83	164.92	110.83	166.99
Harrington	2.04	1.83	0.167	0.174	137.48	201.00	136.72	201.35
OSU_060	2.07	1.65	0.178	0.190	137.42	196.01	136.46	195.95
OSU_052	2.44	1.92	0.174	0.174	152.49	214.54	154.46	217.49
OSU_144	2.65	2.52	0.155	0.129	161.56	242.58	159.86	242.46

Table 2. Estimated root growth parameters for lateral roots using the optimisation pipeline (Figure 2) and comparison between measured and predicted total root length.

Genotype	**Branching** (s^{-1})		**Elongation** $(cm \cdot s^{-1})$		**Total root length**(cm)		**Predicted total root length**(cm)	
	Δt_1	Δt_2	Δt_1	Δt_2	Δt_1	Δt_2	Δt_1	Δt_2
OSU_048	0.383	0.109	0.690	0.260	13.52	37.27	13.42	37.05
OSU_044	0.207	0.114	0.373	0.017	3.59	4.37	7.33	8.27
Harrington	0.442	0.110	0.806	0.087	11.71	20.54	11.52	20.45
OSU_060	0.296	0.110	0.548	0.086	7.07	13.40	7.00	13.67
OSU_052	0.444	0.114	0.814	0.044	7.99	11.91	5.82	9.71
OSU_144	0.337	0.104	0.624	0.168	6.47	19.05	5.37	18.83

Table 3. Analysis of the genotype and time effect on root parameters using a mixed effect model.

Trait	Genotype	Time	Genotype x Time-step
Lateral roots number	ns	***	ns
Lateral total length	*	***	**
Log_lateral_tot_length	ns	***	*
Branching rate	ns	***	ns
Lateral elongation rate	ns	*	ns
Log_lateral_elong_rate	*	ns	**
Seminal roots number	ns	ns	ns
Seminal elongation rate	***	***	***

Statistical significance (p-values) are provided for the fixed effects using a chi-squared based Wald-test using residual maximum likelihood (REML). Level of significance is provided for (*) $p<0.05$; (**) $p<0.01$; and (***) $p<0.001$.